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by

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JULIA MARGRETHE FOGHT ROBINSON

A THESIS

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Date.....August 19, 1985.....

Abstract

Mixed bacterial crude oil-degrading cultures of marine and terrestrial origin were screened for pure isolates capable of degrading polycyclic aromatic hydrocarbons (PAH). All but one isolate exhibiting PAH degradative ability carried at least one plasmid.

Three marine isolates with differing degradative capabilities were selected, subjected to taxonomic tests, and tentatively identified as *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1). Their degradative capabilities were examined using gas chromatography, radiorespirometry and thin-layer chromatography with autoradiography to observe degradation products, and using culture techniques to determine growth on PAH. The *Flavobacterium* (8W-2) isolate was chosen for intensive study because it was able to metabolize a wide variety of PAH. Plasmids from the isolates were visualized using modifications of published plasmid isolation procedures; *Acinetobacter* (8W-1) carried 4 small plasmids, *Flavobacterium* (8W-2) carried 3 medium-sized plasmids, and *Aeromonas* (38W-HC10-1) carried one large plasmid.

Using chemical agents and physical/biochemical curing techniques, phenotypic variants of the isolates were generated. Because of technical limitations, initial screens were indirect, employing antibiotic- and metal-resistance; as it became apparent that these traits were not linked to PAH degradation, direct screens for PAH metabolism were

used. The extent of phenotypic variation was examined using ^{14}C-labelled PAH for mineralization studies and spectrophotometry for identification of pathway intermediates. Phenanthrene was found to be completely mineralized by *Flavobacterium* (8W-2) via the o-phthalate pathway, and naphthalene was mineralized by the salicylate pathway. Dibenzothiophene and anthracene were co-oxidized, following published degradation pathways. Growth studies using both PAH and pathway intermediates as sole carbon sources provided information regarding regulation of the degradative pathways.

Flavobacterium (8W-2) plasmids exhibited a unique *in vitro* instability which was unexplained, but may be a function of reversible changes in tertiary plasmid structure, or associations with macromolecules. A limited number of restriction enzyme digests and hybridization studies revealed a lack of homology of 8W-2 and 38W-HC10-1 plasmids with TOL pWW0.

Correlation of PAH phenotype with plasmid content was difficult, since complete curing of the isolates was not achieved. Phenotypic variants of *Acinetobacter* (8W-1) and *Flavobacterium* (8W-2) demonstrated altered plasmid profiles which implied plasmid involvement in PAH degradation. Failure to observe any plasmid changes in *Aeromonas* (38W-HC10-1) phenotypic variants suggested a chromosomal location for its limited degradative capability, but plasmid involvement could not be excluded. Assignation of degradative functions to specific plasmids was complicated by the observation of apparently constitutive degradative activities, which presumably were due to chromosomal non-specific oxygenases or to redundant isofunctional enzymes, and by the presence of multiple plasmids in *Flavobacterium* (8W-2) and *Acinetobacter* (8W-1).

Unfortunately, conjugation and transformation studies were unsuccessful with intrageneric recipients; isogenic recipients could not be generated because of incomplete curing. It is not clear whether this failure was due to technical reasons for lack of transfer, or to biochemical/ genetic reasons for lack of phenotype expression. The latter concern is significant in view of the probability of chromosomal involvement in plasmid-mediated PAH degradation.

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Abbreviations

d	day(s)
hr	hour(s)
min	minute(s)
sec	second(s)
ASW	Artificial Sea Water medium
BMA	Basal Marine Agar
BME	β-mercaptoethanol
BSA	bovine serum albumin
CCC	covalently closed circular (DNA)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Mdal	megadalton(s)
NP	nitrogen and phosphorus supplement
OC	open circular (DNA)
PAH	polycyclic aromatic hydrocarbon(s)
PB	Prudhoe Bay crude oil
PBa	Prudhoe Bay crude oil, aromatic fraction
PCA	Plate Count Agar
SDS	sodium dodecyl sulfate
STCS	salty trypticase soy broth
TE	Tris-EDTA buffer
TEAS	Tris-EDTA-sodium acetate-NaCl buffer
TEB	Tris-EDTA-boric acid buffer
TES	Tris-EDTA-NaCl buffer
t.o.d.	tetraazotized-o-dianisidine
Tris	tris(hydroxymethyl)aminomethane
YE	yeast extract

Phenotypes:	Bph	biphenyl degradation
	Dbt	dibenzothiophene degradation
	Nah	naphthalene degradation
	Phn	phenanthrene degradation
	Sal	salicylate degradation
	m-Tol	m-toluate degradation

Plasmids:	DBT	dibenzothiophene degradative plasmid
	NAH	naphthalene degradative plasmid(s)
	PHN	phenanthrene degradative plasmid
	SAL	salicylate degradative plasmid
	TOL	toluene/xylenes degradative plasmid(s)

Chapter 1

Introduction

1.1 Definition and sources of polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are defined as chemicals composed of only carbon and hydrogen in fused benzene-ring structures with a minimum of two (Cripps and Watkinson, 1978) or three (Edwards, 1983; Blumer, 1976) units. They may be arranged in linear, angular or clustered arrays (Figure 1.1). Nitrogen, sulfur, and oxygen atoms can be substituted readily into these rings, producing 'heteroaromatic' compounds (Blumer, 1976) which are often grouped with PAH because of their similar chemical and physical properties, and because they often exist in the same sources as PAH; for example, petroleum.

Sources of PAH include forest fires, volcanoes, synthesis by some plants (Edwards, 1983; Suess, 1976), and anthropogenic activities such as incomplete combustion of fossil fuels, jet aircraft exhaust, coal gasification plants, petroleum spills, and the exhaust from internal combustion engines (Edwards, 1983).

1.2 Chemical Degradation of PAH

The main decomposition process for PAH on a global scale is photo-oxidation (Bartell *et al.*, 1981). PAH will also react with nitrogen oxides and sulfur oxides in the

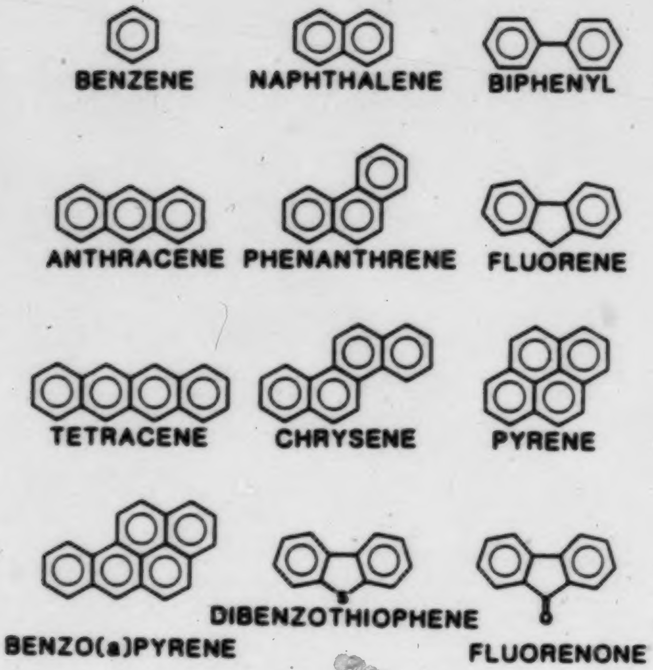
BENZENE
NAPHTHALENE
BIPHENYL
ANTHRACENE
PHENANTHRENE
FLUORENE
TETRACENE
CHRYSENE
PYRENE
BENZO(a)PYRENE
DIBENZOTHIOPHENE
S
O
FLUORENONE

Figure 1.1 Structures of some aromatic compounds.

atmosphere to produce mutagenic compounds in addition to forming endoperoxides which ultimately become direct-acting mutagenic quinones (Edwards, 1983).

The thermochemical stability of PAH varies according to arrangement of the benzene units. According to Blumer (1976), the linear arrays of anthracene and tetracene (Figure 1.1) are the least stable. Of intermediate thermochemical stability are the clustered benzene rings such as pyrene and benzopyrene, and the most stable configurations are the angular clusters found in phenanthrene and chrysene.

1.3 Mammalian Alteration of PAH

Many PAH have demonstrated toxicity, mutagenicity, and carcinogenicity in higher life forms. Eleven of forty PAH listed by the National Academy of Sciences are regarded as strongly carcinogenic or mutagenic, and ten are listed as weakly mutagenic or carcinogenic (National Academy of Sciences, 1972; cited by Edwards, 1983). The well-known PAH such as benzo(a)pyrene often are not toxic themselves, but may be activated by biological oxidation to epoxides which are strongly carcinogenic. The metabolism of PAH in mammals via mixed function oxidases has been studied in some detail (Wood *et al.*, 1977; DePierre and Ernster, 1978; Thakker *et al.*, 1980). Researchers have found that cytochrome P-450 involvement in mammalian microsomal detoxification reactions leads to the formation of arene oxides by incorporation of one atom of oxygen into the PAH ring (Cripps and Watkinson, 1978). The arene oxides may subsequently form *trans*-dihydrodiols, phenols or dihydrodiol epoxides of varying carcinogenicity and teratogenicity (Cerniglia, 1984).

1.4 Microbial Degradation of PAH

Although there are many species of bacteria, fungi and yeasts capable of degrading a variety of fossil fuel hydrocarbons (Cerniglia and Perry, 1973; Cerniglia and Crow, 1981; Atlas, 1981), many PAH tend to be resistant to microbial degradation because of their condensed rings. They therefore should have the potential to accumulate in the environment. The fact that they do not accumulate as predicted is probably due to microbial degradation, which is the second major fate of PAH (Suess, 1976).

In contrast to mammalian metabolism, bacterial oxidation of PAH proceeds via incorporation of both atoms of molecular oxygen into the ring via dioxygenases, to yield *cis*-dihydrodiols (Jerina *et al*., 1976). The products of bacterial metabolism are considered less toxic than those of mammalian metabolism, whereas yeast and fungal metabolites resemble mammalian products (Cerniglia, 1982; Cerniglia and Gibson, 1979). The chemical stability of PAH previously referred to is at odds with microbial degradation observations, which show that phenanthrene is one of the easiest PAH to degrade, anthracene more difficult, and benzo(a)pyrene and chrysene much more difficult for bacteria to degrade (Herbes and Schwall, 1978; Dean-Raymond and Bartha, 1975). In fact, it appears that compound size is related to degradability, since naphthalene is more readily degraded than the larger compound anthracene, which in turn is more easily degraded than benz(a)anthracene (Herbes and Schwall, 1978). Herbes and Schwall (1978) state that "the ease of transformation of PAH by acclimated microorganisms is inversely related to the number of rings." It is probable that this phenomenon is related to the solubility of the compounds. Wodzinski and Bertolini (1972) and Wodzinski and Coyle (1974) have demonstrated that the physical state of the hydrocarbons determines utilization; that is, the PAH must be utilized in the dissolved state by bacteria. As solubility decreases with PAH size, it is probable that the limiting chemical factor in biodegradation is the amount of soluble PAH available for bacterial attack (Groenewegen and Stolp, 1976; cited by Cripps and Watkinson, 1978).

The extent of bacterial attack on PAH is varied. The smaller PAH (e.g. biphenyl, naphthalene and phenanthrene) may serve as carbon sources, being completely mineralized by certain bacteria. A composite degradative scheme summarizing the most common pathways used by PAH-degrading bacteria is presented in Figure 1.2. Of the PAH which are completely degraded, it is interesting to note the convergence of the different degradative pathways to common intermediates. Some compounds (e.g. anthracene, and the heterocycle

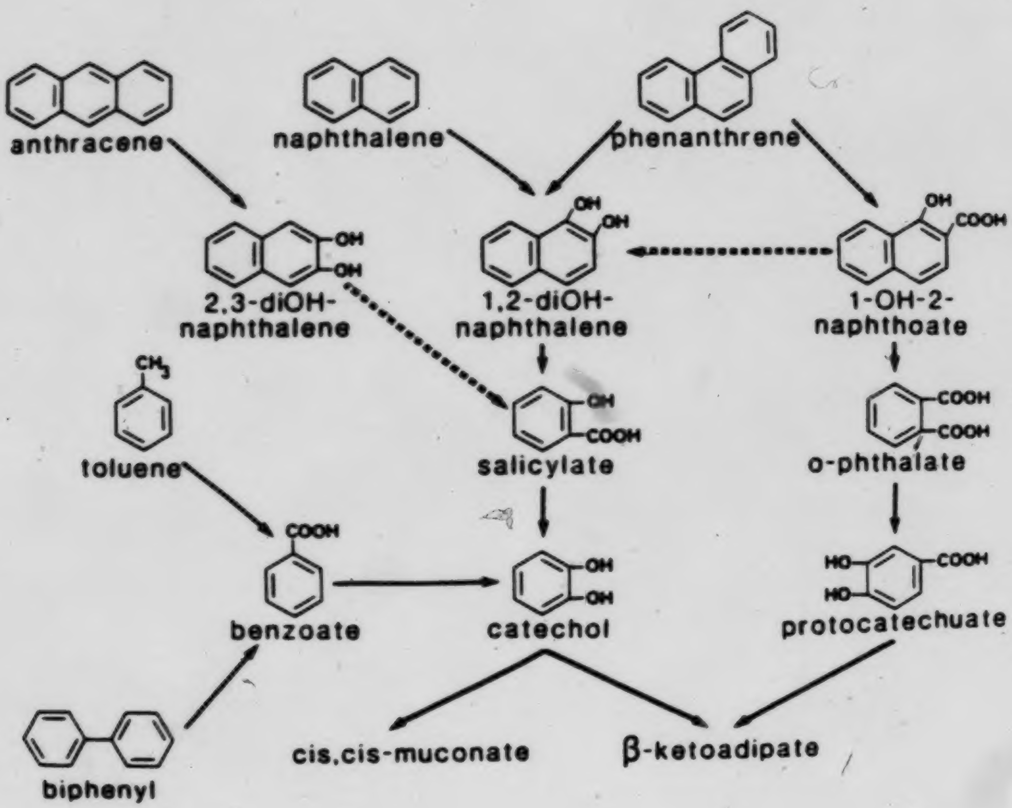
anthracene
naphthalene
phenanthrene
2,3-diOH-naphthalene
1,2-diOH-naphthalene
1-OH-2-naphthoate
toluene
salicylate
o-phthalate
benzoate
catechol
protocatechuate
biphenyl
cis,cis-muconate
β-ketoadipate

Figure 1.2 Degradative pathways commonly used by PAH-degrading bacteria. Selected intermediates are illustrated. A broken line indicates that the pathway is used by a limited number of species.

dibenzothiophene, not illustrated) are partially metabolized, undergoing limited ring cleavage and producing apparently recalcitrant final products; such compounds may not serve as a carbon source but usually are metabolized through co-oxidation (Cripps and Watkinson, 1978; Finnerty *et al.*, 1983). PAH with more than three rings appear to be subject to limited oxidative attack, and usually do not experience ring cleavage; they have never been reported to be capable of sustaining bacterial growth (Gibson and Subramanian, 1984).

1.5 Degradative Plasmids

Although the microbial degradation of aromatic hydrocarbons has been known for many years, the discovery of plasmid involvement is recent. A series of papers in *The Journal of Bacteriology* and *Proceedings of the National Academy of Sciences* in a 3 year span presented the first circumstantial evidence for the existence of catabolic plasmids encoding degradative genes for: salicylate,"SAL" (Chakrabarty, 1972); D-camphor, "CAM" (Rheinwald *et al.*, 1973); *n*-octane, "OCT" (Chakrabarty *et al.*, 1973); naphthalene, "NAH" (Dunn and Gunsalus, 1973); and toluene, "TOL" (Williams and Murray, 1974). After this spate of papers, it was only a short while until new plasmids related to TOL were discovered: "XYL" (Friello *et al.*, 1976), and a family of TOL plasmids (Williams and Worsey, 1976). It was considerably longer before circumstantial evidence for plasmids involved in PAH degradation were reported: phenanthrene degradation (Kiyohara *et al*., 1981) and dibenzothiophene oxidation (Finnerty *et al*., 1983; Monticello *et al*., 1985a), which will be discussed later.

The term "degradative plasmids" was coined by Chakrabarty in 1973 to designate the naturally occurring plasmids like NAH, SAL, and OCT and to differentiate them from the fertility (F-) plasmids and drug-resistance (R-) plasmids. Initial evidence for the existence of these degradative plasmids and their involvement in aromatic degradation was based on the loss of the specific degradative phenotype and subsequent low reversion frequencies after typical curing treatments were applied, and on conjugal transfer of phenotypes, rather than on physical evidence for the plasmids. These degradative plasmids were initially proposed to exist in *Pseudomonas* spp., but their visualization had to await modification of methods designed for *E. coli* plasmids. Coincident with genetic studies were enzymatic and nutritional studies which eventually led to the large volume of literature dealing with the best understood of the degradative plasmids, TOL. Almost all of these degradative plasmids have been studied in one or another of the *Pseudomonas* species, usually *P. arvilla* mt-2, *P. putida*, or *P. aeruginosa*.

Regarding the degradative plasmids of *Pseudomonas*, a few general observations can be made.

1. They are usually large plasmids, greater than 35 Mdal

(Haas, 1983).

2. The plasmids are usually transferrable by conjugation or transformation to other related pseudomonads, but not to other genera (Chakrabarty, 1976).
3. The enzymes for a pathway are often clustered, with "supra-operonic" clustering occuring less frequently (Haas, 1983).
4. The plasmids encompass several different incompatibility groups (Boronin *et al.*, 1980), notably IncP2 (CAM, OCT) and IncP9 (NAH, SAL, TOL).
5. Degradative plasmid genes not uncommonly interact with chromosomally-encoded degradative genes (Haas, 1983; Bayly and Barbour, 1984).

Because of the paucity of literature reports on PAH plasmids, a review of plasmids involved in the degradation of smaller aromatic compounds is presented here. For further information, the reader also is referred to recent reviews by Haas (1983), Singer and Finnerty (1984), Cerniglia (1984), and Gibson and Subramanian (1984).

1.5.1 The TOL family of plasmids

The TOL family of plasmids found in *Pseudomonas* spp. includes an isofunctional group of at least 30 morphologically diverse, physically distinct plasmids, which encode genes for the degradation of toluene, m-toluate and p-toluate. TOL plasmids (hereafter referred to simply as TOL) are widely distributed in soil *Pseudomonas* isolates

(Duggleby *et al.*, 1977).

The enzymes specified by genes on TOL are relatively non-specific and are responsible for the oxidation of toluene and m- and p-xylenes to their corresponding alcohols, and for the oxidation of benzyl alcohol and m- and p-methylbenzyl alcohols to benzoate and m- and p-toluate, respectively (Worsey and Williams, 1975; Figure 1.3). The enzymes responsible for the upper portion of the pathway (i.e. aromatic hydrocarbons oxidized to benzoic acid) and the lower pathway (i.e. degradation of benzoic acid) appear to be organized into two operons with two regulatory genes (Figure 1.3). The interaction of the xylR and xylS gene products with coinducer pathway substrates induces the degradation of toluene and xylenes via *meta*-cleavage of catechol. In addition, in most *Pseudomonas* host cells, there is a set of chromosomal genes encoding enzymes for *ortho*-cleavage of catechol. However, the regulatory system for the archetypal TOL plasmid, pWWO, is such that the *meta*-cleavage pathway is dominant when induced by toluene or related compounds like m- or p-toluate or m- or p-xylene. Strangely, the *ortho*-cleavage pathway is more energetically favorable but is repressed unless the bacteria are grown with benzoate as the sole carbon source. This presents a simple method for isolating *Pseudomonas* cured of TOL: in a minimal salts-benzoate medium, cured strains will outgrow those plasmid-containing strains confined to the *meta*-cleavage pathway.

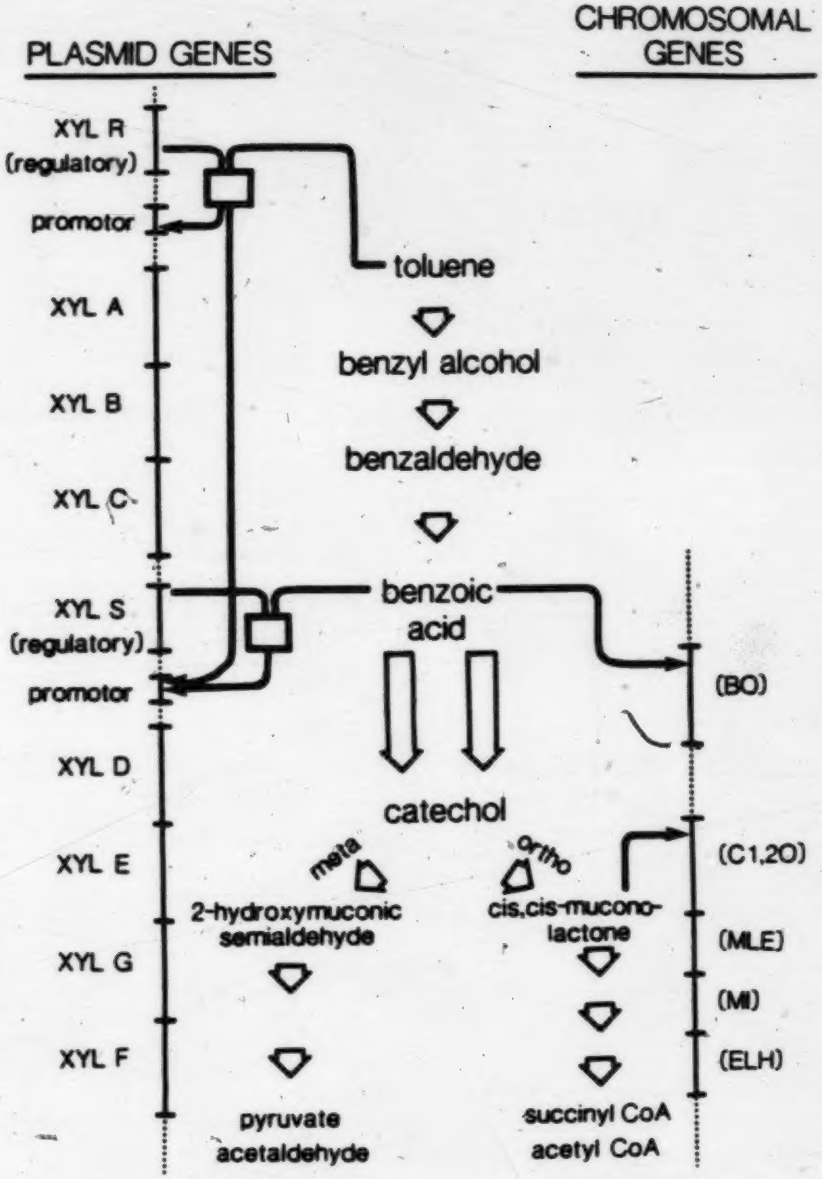
PLASMID GENES
CHROMOSOMAL GENES
XYL R (regulatory)
promotor
XYL A
XYL B
XYL C
XYL S (regulatory)
promotor
XYL D
XYL E
XYL G
XYL F
toluene
benzyl alcohol
benzaldehyde
benzoic acid
catechol
meta
ortho
2-hydroxymuconic semialdehyde
cis,cis-muconolactone
pyruvate
acetaldehyde
succinyl CoA
acetyl CoA
(BO)
(C1,2O)
(MLE)
(MI)
(ELH)

Figure 1.3 Degradation of toluene via benzoic acid by *Pseudomonas putida* arvilla mt-2 carrying the TOL plasmid. Solid lines indicate positive regulation. The same enzymes degrade m- and p-xylenes via their corresponding acids (not illustrated). Adapted from Williams and Murray (1974), Shapiro *et al*. (1980), Franklin *et al*. (1981) and Singer and Finnerty (1984).

The TOL family of plasmids exhibits a number of interesting characteristics, one of which is the occurrence of spontaneous deletions or insertions. Some TOL plasmids have been shown to undergo non-specific excision events when grown in non-selective media (Pickup and Williams, 1982). Other TOL plasmid-containing strains grown in selective media (e.g. m-toluate) demonstrated a heterogeneous plasmid population with sizes ranging to 200 Mdal, whereas a single species of plasmid was observed when the same strain was grown in nutrient broth (Williams, 1978; Duggleby *et al.*, 1977). This phenomenon may be related to the observation that some TOL plasmids undergo precise excisions of 30 Mdal (Bayley *et al.*, 1977; Kunz and Chapman, 1981), separating the plasmid into a degradative portion which is non-conjugative, and a conjugative portion which does not encode degradative genes (Chakrabarty *et al.*, 1978). This excision is apparently mediated by segments of direct repeats (Jeenes and Williams, 1982; Lehrbach *et al.*, 1982) analogous to transposable elements. Observation of heterogeneous TOL plasmid populations within a strain may then be a reflection of this transposition capability. The TOL plasmids also have been observed to transpose into other plasmids (Jacoby *et al.*, 1978) and into the chromosome (Williams *et al.*, 1980; Chatterjee and Chakrabarty, 1982; Meulien and Broda, 1982) again presumably through transposon-like repeats.

Study of the TOL plasmids' functions is complicated by the fact that TOL plasmids from different sources are distinct, often with unique restriction digest patterns (Duggleby *et al*., 1977). In fact, many of the apparently conflicting reports regarding properties of TOL are due to the use of different hosts. A number of host-specific effects have been reported, such as curing efficiencies (Kunz and Chapman, 1981), temperature-sensitive plasmid maintenance (Singer and Finnerty, 1984), success of isolation (Pickup and Williams, 1982) and restriction digest patterns (Singer and Finnerty, 1984). In addition to these phenomena, there have been observations of cooperativity between plasmid-specified and chromosomally-encoded metabolic activities (Jeenes *et al*., 1982), which further complicates the study of plasmid-borne functions.

There are genetic and functional similarities between TOL and the non-conjugative XYL plasmid encoding degradation of m- and p-xylenes; presumably the regulation of XYL functions is similar to TOL (Singer and Finnerty, 1984). However, XYL does not appear to belong to the IncP1, IncP2 or IncP9 incompatibility groups. There is also some sequence homology between TOL and other degradative plasmids such as SAL and NAH (Heinaru *et al*., 1978).

1.5.2 NAH Plasmids

The NAH plasmids encode some or all of the genes necessary for degradation of naphthalene through salicylate. In some NAH plasmids, the degradative genes are divided into two operons corresponding to the upper and lower pathway enzymes, as with the TOL system; that is, the nah1 operon encodes degradation of naphthalene to salicylate and the nah2 operon encodes degradation of salicylate through *meta*-cleavage of catechol (Austen and Dunn, 1980; Yen and Gunsalus, 1982). A regulatory region resides between the two operons. However, in some *Pseudomonas* strains, there is no evidence for plasmid-encoded degradative functions and the nah genes are presumably chromosomal (Dunn and Gunsalus, 1973; Connors and Barnsley, 1982). In still other strains, the degradation of naphthalene appears to result from cooperation between plasmid- and chromosomally-encoded genes (Zuniga *et al.*, 1981; Dunn *et al.*, 1980; Cane and Williams, 1982) such that the upper pathway enzymes are chromosomal and the lower ones are plasmid-mediated.

A variety of regulatory schemes have been reported for different NAH plasmids in different hosts. For example, Williams *et al.* (1975) found that growth of *Pseudomonas* spp. on naphthalene induced all degradative enzymes of the naphthalene pathway and that growth on salicylate induced the catechol *ortho*-cleavage enzymes but no naphthalene degradative genes; Barnsley (1975) found that, in a different *Pseudomonas* strain, growth on salicylate induced all the naphthalene degradative pathway enzymes; Austen and Dunn (1980) studied three strains, all identified as NCIB 9816, but which demonstrated three different regulations of naphthalene catabolism, dependent upon induction. There are differences between NAH plasmids within the same strain, also: Connors and Barnsley (1982) described plasmid NAH2 which produced constitutive low levels of catechol *meta*-cleavage enzymes, while plasmid NAH3 produced constitutive high levels of the same enzyme; Barnsley (1976) also found different patterns of regulation in strains of *Pseudomonas* which contained apparently identical NAH plasmids. Some of these differences undoubtedly can be attributed to interference by the aforementioned chromosomal influences via redundant degradative genes (Singer and Finnerty, 1984).

However, NAH plasmids which appear to be identical in molecular weight on agarose gels may actually be different. Connors and Barnsley (1982) reported that three NAH plasmids which co-migrated in agarose gels demonstrated sizes of 52 Mdal (NAH) and 77 Mdal (NAH2, NAH3); the larger plasmids presumably were more highly supercoiled. The NAH2 and NAH3 plasmids, although isofunctional with the archetypal NAH plasmid, also exhibited distinctly different digest patterns (which hybridized extensively with NAH).

Connors and Barnsley (1982) also reported that host-specific effects influenced both curing and isolation of NAH plasmids in certain *Pseudomonas* strains, and Cane and Williams (1982) reported physical modifications to NAH plasmids transferred to alternate *Pseudomonas* hosts.

NAH plasmids are closely related to SAL plasmids (Heinaru *et al*., 1978), but SAL is not a simple excision derivative of NAH. Both SAL and NAH plasmids show less homology with TOL. In a manner similar to the TOL system, some NAH plasmids have been observed to acquire site-specific insertions and deletions of DNA (Cane and Williams, 1982).

1.5.3 Phenanthrene plasmids

Phenanthrene was shown to be degraded via o-phthalate by an *Aeromonas* sp. (Kiyohara *et al*., 1976), in contrast to a previous report by Evans *et al*. (1965) that phenanthrene was degraded by pseudomonads via the naphthalene pathway intermediates salicylate and catechol. The pathways for phenanthrene and naphthalene degradation subsequently were found to be separately encoded and regulated in 13 soil bacterial isolates (Kiyohara and Nagao, 1978). Despite this knowledge of the catabolism of phenanthrene, Kiyohara *et al*. (1981) did not report the isolation of putative PHN plasmids until a 1980 conference, when circumstantial evidence was presented that a 44 Mdal (pHK1) and/or a 22 Mdal (pHK2) plasmid encoded genes for phenanthrene degradation in *Alcaligenes faecalis* AFK2. The strain could be cured of one or both plasmids by incubation with chemical curing agents, or with o-phthalate as sole carbon source (as a parallel with benzoate curing of TOL). Loss of one or both plasmids was not absolutely correlated with the Phn$^-$ phenotype, and all Phn$^+$ transconjugants contained both plasmids.

Subsequently, Kiyohara *et al*. (1983) reported on plasmid involvement in the degradation of several PAH by a

Beijerinckia sp. which contained two plasmids: pKG1 (147 Mdal) and pKG2 (21 Mdal). Although chemical curing agents were ineffective, loss of the smaller plasmid by subculture in benzoate led to a Phn⁻ Bph⁻ phenotype, suggesting that pKG2 was involved in degradation of phenanthrene and biphenyl. Unfortunately, all conjugation and transformation attemps were unsuccessful with either isogenic or intrageneric recipients and so pKG2 remained a putative PHN plasmid.

A report by Ghosh and Mishra (1983) assumed plasmid involvement in Phn⁺ and Nah⁺ phenotypes of a *Micrococcus* sp. because of the observation that the phenotypes were lost after subculture under non-selective conditions. However, the Phn⁺ phenotype was not lost by subculture with o-phthalate as sole carbon source, and no plasmids were ever isolated.

Therefore, no unequivocable evidence has been presented that phenanthrene degradation is plasmid-encoded; yet there is circumstantial evidence for this supposition, and indeed, there is no reason why phenanthrene metabolism should not be plasmid-mediated, given the existence of toluene, biphenyl, and naphthalene degradative plasmids.

1.5.4 Other degradative plasmids

It was suggested by Finnerty *et al*. (1983) that oxidation of dibenzothiophene by specific *Pseudomonas* spp. was mediated by plasmids. In a recent publication, Monticello *et al.* (1985a) reported results of curing and conjugation studies indicating that a 55 Mdal plasmid was responsible for cooxidation of dibenzothiophene in 2 of 3 soil *Pseudomonas* isolates; in the third isolate, no plasmid was found. The metabolism of naphthalene and salicylate by the two plasmid-bearing isolates also was presumed to be plasmid-mediated. Theirs is the first definite report of plasmid-encoded dibenzothiophene metabolism.

A variety of other compounds, both natural and synthetic, have been shown to be degraded via plasmid-mediated pathways, including chlorinated biphenyls (Furukawa and Chakrabarty, 1982), herbicides such as 2,4-D (Fisher *et al.*, 1978; Pierce *et al.*, 1981) and nylon oligomers (Negoro *et al.*, 1980).

A considerable amount of effort has been devoted to the study of the alkane degradative genes encoded by the OCT plasmid (as reviewed by Singer and Finnerty, 1984), which is complicated by the presence of redundant chromosomal genes. Similar combined chromosomal and plasmid degradation has been observed with phenol degradation (Wong and Dunn, 1976) and camphor oxidation (Rheinwald *et al.*, 1973) in *P. putida*. Chromosomal involvement in plasmid-mediated degradative functions may include regulatory (induction) aspects, production of isofunctional but separately regulated enzymes, chromosomal integration/excision phenomena, or plasmid mobilization of chromosomal genes (Haas, 1983; Hughes *et al.*, 1984). These phenomena significantly complicate the task of assigning phenotypic functions to specific plasmids.

It should be apparent from this brief review that the majority of information on degradative plasmids has been published in the last five years. When this project was begun in 1980, there was a need to study the role of plasmids in degradation of PAH to supplement information concerning the small aromatic compounds (e.g. toluene, salicylate, naphthalene). In fact, there is still a lack of published information about the PAH degradative plasmids, but this gap is being filled by recent publications on PHN (Kiyohara *et al*., 1983) and DBT plasmids (Monticello *et al*., 1985a,b). It has become apparent that mere extrapolation from the TOL plasmid system to PAH plasmid systems is inappropriate, although the complexities of regulation (Figure 1.3), excision, integration, etc. of the TOL system provide a useful model for study of the PAH plasmids.

1.6 Objectives

The immediate purpose of this project was to study the degradation of specific PAH by environmental isolates, and if possible to ascertain whether these degradative capabilities were plasmid-mediated. The long-range, idealistic goal was to contribute to the knowledge necessary to enable genetic engineering of organisms, enhancing their ability to degrade recalcitrant pollutants such as PAH. Survival of such engineered organisms in the environment is necessary for successful introduction of PAH degraders; hence the choice of environmental isolates for this project rather than culture collection isolates. As the scientific literature has expanded current knowledge regarding the complexities of plasmid-mediated aromatic and PAH degradation, this choice of environmental isolates was shown to be premature, since most extant genetic techniques were developed for a limited number of culture collection isolates, particularly *E. coli* and *Pseudomonas* spp. Similarly, as reports from other laboratories were published during the course of this thesis, many initial assumptions regarding the PAH degraders were shown to be naive. However, with the rationale that plasmid involvement in bacterial PAH degradation was plausible, this project attempted to correlate plasmid content with PAH degradative capability, which necessitated defining the PAH phenotype and characterizing the plasmid genotype. Several approaches were used, including simple genetic and phenetic screening, attempted plasmid curing, isolation of degradative pathway mutants, and attempted conjugation and transformation.

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Chapter 2

Isolation, Taxonomy and Growth of PAH-Degrading Bacteria

2.1 Introduction

Historically, microbes capable of degrading PAH such as phenanthrene, naphthalene and dibenzothiophene have been isolated from environmental samples by enrichment on the substrate of interest (Dunn and Gunsalus, 1973; Kiyohara *et al.*, 1983; Finnerty and Hartdegen, 1984). Since crude oil contains a wide variety of PAH, environmental mixed cultures capable of oil degradation presumably contain PAH-degraders.

A number of crude oil-degrading mixed cultures of marine or terrestrial origin have been maintained in this laboratory for several years by transfer in medium containing crude oil as sole carbon source. These cultures, in addition to new mixed cultures enriched by growth on Prudhoe Bay aromatics fraction (PBa), were used as sources of PAH-degrading bacteria. The mixed cultures were separated into pure isolates which were grown with crude oil and screened by gas chromatography (GC) for degradation of specific PAH.

Pure isolates capable of PAH degradation were subjected to a number of taxonomic tests, including colonial and cellular morphology, motility, presence of catalase and oxidase activity, and anaerobic and aerobic growth on glucose plates. Some strains were subjected to further taxonomic tests, including antibiotic and heavy metal resistance, presence of flagella and capsules, various enzymatic and biochemical activities, extracellular DN'ase activity, electron microscopy and partial characterization of pigments. Results from these tests allowed tentative identification to the genus level.

Three marine isolates identified as strains of *Flavobacterium*, *Aeromonas*, and *Acinetobacter* genera were chosen for further degradation studies employing radiolabelled PAH. Mineralization was monitored by measuring production of $^{14}CO_2$ and partial degradation of substrates was observed by autoradiography.

The need for large quantities of cell material for plasmid isolation and characterization was anticipated. Therefore, various supplements to minimal media were tested in studies designed to increase cell yields of the three chosen isolates. Growth in minimal medium with PAH as carbon source was considered essential for two reasons: first, the organisms had originally been isolated from low-nutrient environments and were presumably oligotrophic; second, loss of PAH-degradative capability under non-selective conditions (such as in rich medium) was a possibility, similar to the loss of antibiotic resistance in R-plasmid-containing bacteria grown in non-selective media. Eventually, rich media were used to produce cell material and minimal media were used for isolate maintenance.

2.2 Methods and Materials

2.2.1 Isolation and Screening of Pure Cultures for PAH Degradation

Microbial mixed cultures with known oil-degrading capability, designated 38W and SL-PB (Table 2.1), were maintained by monthly transfer at 8°C in Artificial Sea Water plus nitrogen and phosphorus (ASW + NP; Appendix) or at 30°C in Jobson's medium (Appendix), respectively, using crude oil as sole carbon source. Isolates were obtained by serially diluting aliquots of the cultures in sterile ASW (for 38W) or phosphate buffer (for SL-PB) dilution blanks and plating on Basal Marine Agar (BMA; Appendix) or Plate Count Agar (PCA; Difco), respectively. To keep the isolates in contact with hydrocarbons, the plates were spread with either 10μL or 100μL of a synthetic hydrocarbon mixture (Appendix) immediately prior to inoculation. After incubation at 22°C for 1 week, several different colony types were picked from these hydrocarbon plates and streaked for purity onto an appropriate solid medium also spread with the synthetic hydrocarbon mixture. Isolates were given the prefix "HC-10" or "HC-100" in conjuction with site numbers.

Cultures 38W-PBa and SL-PBa were maintained in appropriate liquid media with 0.035%v/v Prudhoe Bay crude oil aromatic fraction (PBa) as the carbon source. The method of preparing PBa by liquid chromatography is presented in the Appendix. This technique was intended to enrich the

Table 2.1 Sources of maintenance cultures for single isolates

Culture	Location	Sample Type	Temp.[1]	Medium[2]	Carbon Source
8W	Point Partridge WA	marine sed.	8°C	ASW+NP	Prudhoe Bay crude oil
20S	Deception Pass, WA	marine sed.	8°C	ASW+NP	Prudhoe Bay crude oil
38W	Duwamish R. WA	estuary water	8°C	ASW+NP	Prudhoe Bay crude oil
38W-PBa	Duwamish R. WA	estuary water	20°C	ASW+NP	Prudhoe Bay aromatics
SL-PB	Shell Lake Alberta	soil	30°C	Jobson's	Prudhoe Bay crude oil
SL-PBa	Shell Lake Alberta	soil	20°C	Jobson's	Prudhoe Bay aromatics

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[1] Temperature of original maintenance culture incubation
[2] Composition of medium in Appendix

original cultures for aromatic degraders. Three colony types prevailed in each culture after four 2-week transfer periods. These isolates were obtained by plating on minimal agar with the synthetic hydrocarbon mixture, and subsequently purifying on BMA or PCA. They were designated "AE", and retained for degradative screening.

In 1981 Ms. R.Clare' separated the component bacterial types of cultures 8W and 20S into 8 pure isolates per culture. These were screened for degradative ability by growing isolates in ASW + NP with Prudhoe Bay crude oil (PB). After a suitable incubation time, the residual oil was extracted with methylene chloride, fractionated by silica gel chromatography into saturate and aromatic fractions, and analyzed by gas chromatography, as per Fedorak and Westlake (1983). She found that, when a degradative function could be ascribed to an isolate, it was either a saturate degrader or an aromatic degrader. Three of her aromatic degraders were considered in this thesis, and are designated 8W-1, 8W-2, and 20S-8.

Individual isolates from the HC-10, HC-100, and AE series as well as from 8W and 20S were screened by incubating in 200mL of appropriate minimal salts medium (ASW+NP or Jobson's) supplemented with 0.1% v/v PB and 0.01% yeast extract (YE). This practice of using a low concentration of supplement has been used by other workers

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'Clare,R. 1981. Microbial degradation of Prudhoe Bay crude oil by isolated species of marine bacteria. 65pp. Project Report for Microbiology 495. Dept. Microbiology, University of Alberta.

(Connors and Barnsley, 1982; Ghosh and Mishra, 1983) to supply trace nutrients and vitamins, and to allow limited growth to occur before degradation begins. After 2 to 3 weeks incubation at 22°C on a rotary shaker at 200 rpm with an eccentricity of 5 cm (standard shaking conditions), the residual oil was extracted with methylene chloride (pesticide grade) and analyzed as per Westlake and Cook (1980). Those isolates showing any degradation of the aromatic fraction, as evidenced by reduction in peak height compared with an uninoculated control, were retained for taxonomy and plasmid screening.

First attempts to use solid media for degradation screens employed minimal agar plates (Appendix) spread with 0.1mL of a pure hydrocarbon solution. The following hydrocarbons were tested: anthracene, carbazole, chrysene, various dimethylnaphthalenes, fluoranthene, 1-methyl- and 2-methylnaphthalene, naphthacene, naphthalene, and phenanthrene. The usual concentrations used were 10μL/mL or 10mg/mL in benzene. Plates were inoculated, the edges wrapped with Parafilm®, and were incubated at 22°C in the dark and scored for growth and/or color production at daily intervals.

Delivery of hydrocarbons to the plates was improved using the technique of Kiyohara *et al*. (1982). In this 'sprayplate' adaptation, anhydrous diethyl ether was used to dissolve a small amount of hydrocarbon. This solution was then sprayed onto an inoculated, unincubated agar plate using a hand-held atomizer such as the compressed air units used for thin-layer chromatography visualization. The hydrocarbon layer thus produced had a finer crystalline appearance, and appeared more uniform than the spread plate. The edges of the plates were wrapped with Parafilm® prior to incubation. Those colonies showing a cleared zone or a colored product surrounding the colony were scored as probable PAH degraders and retained for taxonomic studies.

2.2.2 Taxonomic Studies with Pure Isolates

Morphology

Colonial morphology was observed after growth of the isolates on BMA or PCA incubated at 22°C. Morphology from the HC-10 and HC-100 series of isolates was also performed on BMA or PCA plates spread with 10μL or 100μL of synthetic hydrocarbon mixture. Colony descriptions conform to the ASM manual (Smibert and Kreig, 1981).

Cell morphology was observed with Gram stains after overnight growth in Trypticase Soy Broth (TCS; BBL) or Salty TCS (STCS; Appendix), or after 48 hr on PCA or BMA plates. Observations were made under oil-immersion conditions with a light microscope.

For those isolates giving inconsistent results, or showing Gram variable staining, the KOH procedure was used (Von Graevenitz and Bucher, 1983). A drop of freshly prepared KOH (3% w/v in distilled water) was placed on a clean glass slide and mixed with a loopful of bacterial suspension or liquid culture. If the material became stringy and viscous (due to lysis of the cells), the cells were recorded as Gram negative. If the suspension did not become viscous, but remained milky and particulate, a Gram positive reaction was assumed.

Motility was assessed by the hanging drop method (Doetsch, 1981). A wet mount was prepared from a loopful of 24 hr or 48 hr culture grown in TCS or STCS. Motility was noted using a phase contrast microscope.

The presence of a capsule was determined using the India ink suspension method described in the ASM manual (Doetsch, 1981). A drop of India ink was placed on a glass slide and mixed with a loopful of bacterial suspension. A glass coverslip was placed over half of the suspension and the slide was blotted with tissues to form a thin, even suspension. This was observed with oil-immersion light microscopy. The capsules appeared clear, surrounded by a brown background.

Transmission electron microscope (TEM) studies were performed on cultures grown overnight in STCS then sedimented by centrifugation. Some pellets were gently washed by centrifugation with distilled water to remove salt from the growth medium, which would otherwise crystallize during specimen preparation. Suspensions of cells were placed on formvar-coated copper grids, blotted, and stained with 2% phosphotungstic acid. Dried grids were observed for flagella or other surface structures in the TEM, and photographed on Kodak 35mm Black and White film.

Scanning electron microscope preparations were made from cultures of 8W-2 and 38W-HC10-1 which produced "strands" of gummy material. The strands were carefully removed from the medium and washed gently in a stream of distilled water, then placed in 1.5mL microfuge tubes. The samples were fixed in 5% glutaraldehyde for 2 d at 22°C, then 0.1% osmium tetraoxide for 2 d at 22°C. The fixed strands were washed with ethanol and acetone to dehydrate, and were either observed at this stage, or were further washed with methylene chloride to remove associated hydrocarbons. The prepared strands were placed on aluminum stubs for gold sputter-coating and scanning electron microscopy. Uncoated material was analyzed with an X-ray scanner.

The pigments of isolate 8W-2 were chosen for further study. Aliquots of frozen cells from a 10L STCS culture (Fermenter "B") were used as a source of pigment by mixing thawed cells with 95% ethanol with the aid of a vortex. Extraction into ethanol was followed by centrifugation to pellet the cells. The ethanol supernatant was dehydrated with anhydrous sodium sulfate and subjected to a spectrophotometric scan from 660 nm to 200 nm in a Pye-Unicam double-beam spectrophotometer with a linear recorder, using ethanol as a blank. To test for a bathochromic shift under alkaline conditions (Weeks, 1980b), the sample was then adjusted to 0.01M KOH (final concentration) by the addition of 0.11 volumes of 0.1M ethanolic KOH, and the spectrum was recorded again. The pigments could not be extracted directly into chloroform, so ethanol extracts of the pigments were dehydrated, evaporated under N_2, and re-dissolved in chloroform. This solution was subjected to a spectrophotometric scan as before, using a chloroform blank (McMeekin *et al.*, 1971), and absorption maxima were recorded.

Enzymatic Activities

Several standard tests (ASM manual; Smibert and Kreig, 1981) were performed to determine enzyme activities and growth capabilities.

To test for the presence of cytochrome c oxidase, a piece of filter paper was moistened with a few drops of a freshly prepared solution of tetramethyl-p-phenylenediamine dihydrochloride (1% w/v in distilled water; Sigma). A small portion of a colony from BMA or PCA was smeared on the moistened filter paper. A positive reaction was recorded when a purple color developed on the smeared area within 15 seconds.

The catalase test was performed by suspending part of a colony in distilled water on a glass slide and adding a drop of 3% H_2O_2. A positive reaction was recorded when bubbles appeared due to the cleavage of the peroxide by catalase.

The presence of an extracellular DN'ase was tested by supplementing DN'ase agar (Difco) with a 1% solution of methyl green (0.5% w/v in water, exhaustively extracted with chloroform) and inoculating the agar with a single streak of culture. A positive result was noted by the appearance of a cleared zone around the area of growth. *Staphylococcus aureus* was used as a positive indicator organism.

Gelatin broth, urea broth and starch plates were inoculated with isolate 8W-2 as per the ASM manual (Smibert and Kreig, 1981) to test for gelatinase, urease and amylase, respectively.

Biochemical Capabilities

Standard Oxidative/Fermentative (O/F) plates and O/F plates with salt were prepared (as per Westlake and Cook, 1980; Appendix) using glucose as the carbon source and bromthymol blue as the pH indicator. Plates were incubated at 22°C aerobically or in anaerobic jars, using the Gas-Pak® system (BBL). The presence of visible growth was noted, and the color of the plates (yellow, acid; blue, alkaline; or green, neutral) was recorded at 48 hr for aerobic plates and at 7 d for anaerobic plates.

Standard glucose and lactose broths were used as per the ASM manual (Smibert and Kreig, 1981), and swarming growth plates (Anacker and Ordal, 1959) were inoculated with isolate 8W-2. They were incubated at 26°C for 1 week and scored for acid/gas production or swarming growth, respectively.

Antibiotic resistance was determined in one of two ways.

1. Single isolates were taken from PCA or BMA plates

spread with synthetic hydrocarbon mixture and inoculated into tubes containing 10mL of TCS or STCS with 2 commercial antibiotic discs per tube (Difco or Becton Dickinson). The tubes were incubated at 22°C on a tube roller and scored for resistance by observing turbidity.

2. Bacterial suspensions were plated onto BMA or PCA to obtain a confluent lawn of growth; antibiotic discs were placed on the lawn prior to incubation. The diameters of the zones of growth inhibition were measured in mm and compared with a reference chart provided by Becton-Dickinson (information sheet 08-DPI). Although the standard conditions recommended by Becton-Dickinson were not used, precluding a direct comparison to clinical resistance, the isolates were scored as follows: a zone of inhibition smaller than the value given for clinically resistant isolates was scored as R*, or tentatively resistant; growth of the lawn right up to the antibiotic disc was scored as R, or resistant; a zone of inhibition larger than the reference value was scored as S, or sensitive.

Heavy metal resistance was determined with mercury, cadmium, vanadium and molybdenum by adding filter sterilized solutions of $HgCl_2$ (10µg/mL final concentration), $CdCl_2$ (100µg/mL f.c.), $NaVO_3$ (100µg/mL f.c.), or $NaMoO_4 \cdot H_2O$ (100µg/mL f.c.) to BMA or PCA plates. For qualitative results, a single streak of inoculum was placed on the plate, and the presence of heavy growth after incubation was recorded as resistance.

2.2.3 Increasing Cell Yield

Maintenance cultures of isolates in 200mL ASW + NP + PBa did not produce sufficient cell material for plasmid preparations or inoculum for degradation studies. Therefore attempts were made to increase the yield of cell material and decrease incubation time.

Minimal media

First attempts at increasing cell yield were simply a scale-up of minimal medium from 200mL to 1L volumes with either PB oil or PBa as sole carbon source (2 weeks incubation at 22°C with shaking). However, this scale-up did not reduce the long incubation time, and the presence of residual PB oil and PBa hindered plasmid preparation procedures. Extraction of the hydrocarbons was tried with hexane (which floats on water and does not contaminate the settled cell material), but this treatment tended to lyse the cells before they could be harvested. Hexane is also flammable, making centrifugation hazardous. Extraction with non-flammable methylene chloride (which is more dense than water) contaminated the cell pellet and also lysed the cells.

For this reason, a scaled-up experiment was devised using the relatively insoluble substrates phenanthrene, dibenzothiophene, or naphthalene at 1g/L concentrations on the basis that the unused substrate could be easily separated from spent medium by filtration. This would allow collection of cells in the filtrate by centrifugation.

To effect this experiment, two 2L flasks were connected with sterile glass tubing by short pieces of flexible sterile rubber tubing and one-hole stoppers. One flask contained 1L ASW + NP + 0.01% YE and the hydrocarbon substrate, while the other sterile flask was empty. The empty flask provided additional headspace and oxygen for the closed system and allowed for equilibration and volatilization of hydrocarbons without loss of substrate. The medium was inoculated with cells of isolate 8W-2 grown on BMA, and incubated at 26°C in the dark under standard shaking conditions. After 2 weeks incubation aliquots of the cultures were plated to check for purity. The spent medium was filtered through Whatman #1 paper to remove insoluble substrate, and the filtrate was centrifuged at 5,000 x g for 10 min to sediment bacteria. The amount of cell material collected from 1L of medium after 2 weeks of incubation was sufficient for only 2 or 3 plasmid preparations.

Supplemented media

Further experiments were performed with varying supplements to the minimal medium (ASW + NP) in an attempt to increase cell yield directly, or to increase hydrocarbon degradation thereby increasing cell yield indirectly.

Supplements were added singly or in combinations, and included:

1. nitrogen sources : KNO_3, NH_4Cl, NH_4NO_3, and Nitrogen Base (Difco);
2. carbon sources (0.01 to 0.05% w/v): succinate , acetate, or yeast extract (to 0.1%);
3. trace ferrous iron;
4. solvents: mineral oil or Tween 80;
5. spent medium in sterile dialysis tubing bag placed in fresh medium (in case a low molecular weight inducer of aromatic metabolism was present in spent medium).

Rich media

Attempts were made to increase cell yield using 1/5 strength STCS (dilute STCS; Appendix) with 0.1%v/v PB oil or PBa added after autoclaving. Flasks were incubated for 4 d rather than the 2 weeks required for growth in minimal medium. Eventually, isolate 8W-2 was routinely grown in undiluted STCS for 24 to 48 hr at 26°C, without hydrocarbons.

Large-scale growth

Two fermenter jars (New Brunswick Microferm Fermentors) each containing 10L of STCS were inoculated with 500mL each of a 42 hr culture of isolate 8W-2 (grown from -70°C glycerol stocks) and grown under the following conditions: 4L air/min, 26°C incubation temperature and agitation of 100 rpm. The cultures' optical densities were measured at 600nm as incubation proceeded. One fermenter ("B") was harvested after 24 hr incubation while the other fermenter ("A") was harvested after 42 hr. Cells were harvested by membrane filtration ("Pellicon", Amicon), concentrating 10.5L of culture to approximately 400mL. This volume was then centrifuged at 10,000 rpm (12,100 x g) for 20 min to yield a very soft paste of cells with a volume of approximately 130mL. The paste was divided into aliquots and frozen in plastic scintillation vials at -70°C for plasmid studies.

2.2.4 Degradative Capabilities of Three Isolates

To determine whether isolates 8W-1, 8W-2 and 38W-HC10-1 could actually grow on the PB aromatics fraction as sole carbon source, plate counts were performed using maintenance cultures containing 200mL of ASW + NP and 0.4mL of PBa. A 13% inoculum of 38W-HC10-1 and 8W-2 and a 20% inoculum of 8W-1 were used. (N.B. Inoculum sizes throughout degradation studies were influenced by the degree of growth of the inoculum, which was determined in part by the nutrient status of the growth medium.) The cultures were incubated at 26°C under standard shaking conditions. Aliquots were removed at intervals, diluted in sterile ASW, plated in triplicate onto BMA and incubated at 26°C for 4 d to determine viable numbers.

Individual carbon source studies were conducted as follows.

Isolate 8W-1 and biphenyl mineralization

A suspension of 8W-1 was used to inoculate 50mL of medium in a specially adapted 125mL Erlenmeyer flask (Westlake and Cook, 1980). Four different media were used: (Basal medium = ASW + NP + 0.01% YE)

1. basal medium + 50mg biphenyl + (U-^{14}C)-biphenyl;
2. basal medium + 50µL PBa + (U-^{14}C)-biphenyl;
3. basal medium + 50µL PB + (U-^{14}C)-biphenyl;
4. STCS + (U-^{14}C)-biphenyl.

The exact concentration of ^{14}C-biphenyl added was not calculated, but was in the range of ng/mL, and all flasks received approximately 7,000 dpm. The total biphenyl concentration in medium 1 was approximately 6mM. Uninoculated flasks served as volatility controls. Flasks were sealed with serum stoppers fitted to hold a suspended plastic cup containing pleated filter paper (Westlake and Cook, 1980). The sealed flasks were incubated for 16 d in the dark at 22°C on a rotary shaker at 100 rpm with an eccentricity of 2.5 cm, then sacrificed by acidifying the medium with 2mL of 4N H_2SO_4. The released $^{14}CO_2$ was trapped by adding 0.2mL of Carbo-Sorb II (Packard) to the filter paper and incubating with shaking for 1 hr. The plastic cup containing the filter paper was placed in a scintillation vial containing 10mL of water-compatible fluor (ACS; Amersham). Vials were shaken vigorously, then dark-adapted at 4°C overnight before counting on a Mark III Analytic 6881-C Liquid Scintillation Counter on Program 2, using a microprocessor-controlled program to determine dpm. The amount of (U-^{14}C)-biphenyl mineralized to $^{14}CO_2$ in test cultures was determined by subtracting the values of sterile volatility controls, and dividing by the known activity of (U-^{14}C)-biphenyl added. This produced values of "% label recovered as $^{14}CO_2$".

Isolate 38W-HC10-1 and phenanthrene mineralization

Isolate 38W-HC10-1 was tested for the ability to mineralize (9-^{14}C)-phenanthrene in the same manner as isolate 8W-1, substituting phenanthrene for biphenyl in the procedure outlined above. The isolate was also tested for incomplete oxidation of phenanthrene by autoradiography, using the following procedure.

Isolate 38W-HC10-1 was inoculated into 200mL of ASW + 0.01% YE in a screw-cap Erlenmeyer flask. The medium received 10mg phenanthrene plus approximately 0.5μCi (9-^{14}C)-phenanthrene (≃0.1mg) ±0.2mL PBa. Uninoculated flasks were included as controls. All flasks were incubated at 26°C under standard shaking conditions. Periodically, 10 or 20mL aliquots of the cultures were removed, acidified with 0.25mL conc. HCl, and repeatedly extracted with chloroform. The pooled chloroform extracts were dehydrated with anhydrous Na_2SO_4 and concentrated in a warm water bath under a stream of nitrogen to a volume of approximately 100μL for thin-layer chromatography and autoradiography.

The thin-layer chromatography (TLC) solvent system was derived empirically to separate parent hydrocarbons from their polar metabolites. Naphthalene and 2-naphthol were used as model compounds, and phenanthrene was included as an authentic standard. Stock solutions of these compounds were made in methylene chloride (at 5mg/mL) so that 5µL aliquots were sufficient for visualization under long and short wave ultraviolet light. Unactivated commercial silica gel plates containing a fluorescent indicator (Si250 F plates, 10cm x 20cm; Baker Chemicals) were pre-run to the top with n-hexanes:benzene:methanol (50:50:5) to remove a contaminating compound that migrated with polar solvents. Samples were spotted with a Hamilton microsyringe 1 cm from the bottom edge of the plate and developed in two stages. The primary solvent system (n-hexanes:benzene 95:5) was designed to move the parent, non-polar compounds from the origin. Plates were developed 1.5 hr, or until the solvent front was 18 cm above the origin, and the front was marked. After drying, the plates were observed under both short and long wavelength U.V. light to visualize the reference compounds. The fluorescing areas were lightly marked with pencil for future reference, then a very thin line was scraped clear of silica gel immediately below the areas representing the parent compounds (i.e. phenanthrene, naphthalene). The hydroxylated compounds (i.e. 2-naphthol and phenol) had remained at the origin.Plates were then re-chromatographed in the secondary solvent system (benzene:methanol 98:2) in the same direction until the solvent front almost reached the scraped line. The plates were dried and observed again under short and long wave U.V. light and the fluorescing areas representing polar compounds were marked with pencil. For TLC of radiolabelled compounds, stock solutions of authentic phenanthrene and (9-^{14}C)-phenanthrene were included as U.V. and radiographic markers.

Autoradiography of (9-^{14}C)-phenanthrene-spiked culture extracts was performed by placing the developed TLC plate in contact with Kodak X-ray film in a cassette. Films were exposed for 3 to 5 d at -80°C, then developed conventionally.

The ability of isolate 38W-HC10-1 to grow on phenanthrene in the presence of low levels of yeast extract (as a source of growth factors) was tested by inoculating two flasks containing 200mL of ASW + 0.01% YE. One flask also contained 200mg (≃0.5mM) phenanthrene. A 0.25% v/v inoculum from an overnight STCS culture of 38W-HC10-1 was introduced, and flasks were incubated at 26°C with shaking. Triplicate plate counts on BMA were performed on both flasks at intervals. After 4 d incubation, the phenanthrene-containing flask was supplemented with 2.0mL of standard N,P solution (Appendix), and incubated for a further 9 d. The final pH values of the media were recorded with a glass electrode and a Fisher AccuMet® pH meter.

Isolate 8W-2 growth studies

The ability of 8W-2 to grow on pure single substrates was tested by measuring increases in viable numbers. A 10mL aliquot of a maintenance culture of 8W-2 growing in ASW + NP + PBa was used to inoculate 40mL of ASW + NP containing 5mM biphenyl, phenanthrene, or dibenzothiophene. A flask containing 50μL of PBa was inoculated as a positive control. One inoculated flask was included as a "no carbon" control. The flasks were incubated at 26°C under standard shaking conditions and viable numbers were determined by methods previously outlined.

Mineralization of anthracene was tested by the procedure used for 8W-1 with biphenyl, except that the medium used was ASW + 0.05% yeast extract containing 50μL PBa spiked with either side-(U-^{14}C)-anthracene or (9-^{14}C)-anthracene. An ASW suspension of 8W-2 maintenance culture was used as an inoculum. Uninoculated flasks served as volatility controls. Replicate flasks were prepared, and all flasks were incubated at 22°C in the dark with shaking. At intervals, flasks were sacrificed and the "% label released as $^{14}CO_2$" was determined as previously described.

Mineralization of (7,10-^{14}C)-benzo(a)pyrene, (9-^{14}C)-fluorenone and (9-^{14}C)-phenanthrene were also tested by this method, using ASW with yeast extract or dilute TCS supplements and spiked PBa.

2.3 Results and Discussion

2.3.1 Isolation and Screening of PAH Degrading Isolates

When PAH degrading pure cultures were first isolated, they were maintained in contact with hydrocarbons at all times to provide a positive selective pressure and to preclude the spontaneous loss of degradative capability. The need for selective pressure had been noted by many workers dealing with antibiotic resistance and R plasmids, and by Pierce *et al*. (1981), Kilbane *et al*. (1982) and Furukawa and Chakrabarty (1982), dealing with degradative plasmids. Eventually, BMA was found to be adequate for the pure isolates, and the hydrocarbon plates were no longer used as a transfer medium.

Pure isolates were screened for PAH degradation in two ways: liquid cultures with PB oil or PBa were analyzed individually by GC to ascertain the isolates' range of aromatic degradative capability (Table 2.2); solid medium screening with pure substrates was used as a rapid, simple means of screening several isolates simultaneously. The liquid medium technique had the disadvantage of being slow and labor-intensive. The solid medium screening was of two types: spread plates or sprayplates. A problem with hydrocarbon spread plates is the non-uniformity of the hydrocarbon layer. As the carrier solvent evaporates, thicker areas of hydrocarbon may be left in some places on the plate, making growth interpretation difficult. This

Table 2.2 Some isolates and their degradative capabilities as determined by gas chromatography of extracted residual oil. Isolates incubated in ASW + NP with PB crude oil for 1 to 2 weeks at 24°C. Chromatograms were compared with profiles from parallel uninoculated medium.

Isolate	Aromatic Fraction	Saturate Fraction
8W-1[1]	+[2]	-
8W-2	+++	-
20S-5	-	+++
20S-8	+	-
38W-HC10-1	++	-
38W-HC10-3	-	-
38W-1	-	+++
38W-5	+	-
38W-AE-1	+	-
SL-HC10-1	+	-
SL-HC10-2	-	-
SL-HC10-3	-	-
SL-AE-1	+	-
SL-AE-2	+	-
SL-AE-3	+	-

---

[1] Fedorak and Westlake (1983)

[2] + one or two peaks decreased in height
++ limited degradation
+++ good degradation
- no changes observed in profile

problem is reduced in the sprayplate technique of Kiyohara *et al.* (1982) since the layer of hydrocarbon delivered to the plate is more uniform. Disadvantages to the sprayplate procedure include the following.

1. There is non-quantitative delivery of hydrocarbons to the plate surface; the amount of spray delivered is subjective and quantitatively non-reproducible.
2. Some volatile hydrocarbons like naphthalene and biphenyl tend to volatilize over extended incubation periods, despite the Parafilm® wrap on the plate edges; this necessitates re-spraying the plates periodically.
3. Some larger PAH like chrysene are poorly soluble in diethyl ether, making delivery of a significant amount of hydrocarbon difficult.
4. The aerosol is not sterile; therefore, prolonged spraying (eg. of dilute solutions) sometimes leads to contamination of the plates.
5. Some hydrocarbon derivatives like m-toluate do not produce an opaque layer on the plates because of their water solubility. In the absence of colored metabolites, these substrates are limited to use in selective media, since they are not differential.
6. The colored products of some hydrocarbons like fluorene are transient because of their intermediary nature, so the plates must be scored for color at repeated intervals.

Regardless of the limitations of the procedure, it provides a very quick screen for hydrocarbon-degrading isolates. After initial experimentation, the following hydrocarbons were chosen for routine use in screening because they either (a) have colored oxidation products, or (b) show cleared zones around degrading colonies: phenanthrene, dibenzothiophene, biphenyl and 4-methylbiphenyl, and fluorene. It is assumed that this clearing is due to solubilization or metabolism of the hydrocarbon by the organism and that the colored products represent some metabolite(s) of the hydrocarbon, such as the orange colored products of dibenzothiophene oxidation (Hou and Laskin, 1976; Finnerty *et al.*, 1983), and the yellow and brown colored products of biphenyl oxidation (Fedorak and Westlake, 1983).

On the basis of hydrocarbon degradation screens, taxonomy tests, and plasmid content (Chapter 3), the number of isolates being tested was reduced to three Gram negative isolates of marine origin: 8W-1, 8W-2, and 38W-HC10-1. This simplified the task of media preparation by eliminating the need for a second medium for the terrestrial isolates. Choosing only Gram negative organisms was intended to allow for flexibility in proposed genetic manipulations regarding ease of lysis, conjugation, and transformation. All 3 isolates contained plasmids of different sizes (Chapter 3). The 3 isolates chosen had a range of known hydrocarbon degradative capabilities: 8W-1 was limited to degrading biphenyl and methylbiphenyls (Fedorak and Westlake, 1983);

38W-HC10-1 was capable of limited degradation of smaller molecular weight aromatics such as biphenyl, naphthalenes, and phenanthrene, as determined by gas chromatography; and 8W-2 was capable of the widest range of aromatic degradation of any single organism screened (Table 2.2). For this reason, 8W-2 was used most extensively in these studies.

These isolates have since been maintained in liquid culture by transfer in ASW+NP with PBa as carbon source, at 26°C under standard shaking conditions; on solid medium (BMA) incubated at 26°C and stored at 4°C; and as glycerol-stabilized stocks kept at -70°C.

2.3.2 Taxonomic Studies

Morphology

The colonial morphologies of 12 isolates are summarized in Table 2.3. Although more than 30 isolates were initially used for screening, only a few were chosen for taxonomic identification, and only 3 of these were singled out for detailed examination. The proportion of pigmented colonies included in the taxonomic study is not a misrepresentation of the original populations. It was found that many environmental samples, and especially those with oil-degrading populations, had a high percentage of yellow, orange, or pink pigmented colonies (Westlake and Cook, 1980). One isolate, 8W-2con, was not among the original isolates. It appeared as a contaminant of an 8W-2 culture in minimal medium + PB oil early in the project, and was

Table 2.3 Colonial morphology of several isolates incubated 2 to 7 days at 22°C on BMA or PCA plates. The criteria of Smibert and Krieg (1981) were used for description of morphology.

Isolate	Medium	Description
8W-1	BMA	white, punctiform, convex, entire, <1mm, shiny, translucent
8W-2	BMA	bright yellow, non-diffusing pigment, convex becoming umbonate, entire, 1-2mm, smooth becoming mucoid on wet agar, opaque, butyrous becoming viscous on wet agar
8W-2con	BMA	pale pink, convex, circular, entire, 1-2mm, opaque
20S-8	BMA	tan, opaque, circular, convex, <1mm, entire, dull
38W-O-1	BMA	pale pink, round, pulvinate, entire, 1-2mm, shiny becoming soft, opaque
38W-O-5	BMA	white/colorless, circular, raised, entire, <1mm,shiny, translucent
38W-HC10-1	BMA + HC mix	tan, flat becoming umbonate, undulate, 3mm, wet, shiny, translucent
38W-HC10-3	BMA + HC mix	white, circular, pulvinate to umbonate, entire, 1-2mm, soft, opaque
SL-HC10-1	PCA + HC mix	creamy yellow, circular, pulvinate, entire, 4-6mm, opaque
SL-HC10-2	PCA + HC mix	colorless, pulvinate, circular, entire, 1-2mm, translucent
SL-HC10-3	PCA + HC mix	bright yellow, non-diffusible pigment, convex, circular, entire, 1.5mm, opaque

retained since it had some degradative capability. At the beginning of the project, most isolates were slow growing on PCA or BMA. For example, 8W-2 required about 4 d at 22°C to show significant growth, while 8W-1 required at least 7 d. As the project progressed, it was noted that the incubation times required for both plates and broths decreased for most isolates.

Cell morphologies and Gram reactions are presented in Table 2.4. Motility, as observed in hanging drop preparations, is also recorded in Table 2.4. The majority of isolates were Gram negative rods, which is common in marine water samples (Westlake and Cook, 1980).

The cells of isolate 8W-2 were observed for capsules by the India ink method, and were found to have a colorless capsule-like area surrounding the cells. Isolate 38W-HC10-1 also demonstrated this capsule when stained, but to a lesser extent than isolate 8W-2.

To observe the cells, their capsules, and to look for surface structures, transmission electron microscopy was used. Isolate 8W-1 stained evenly, and showed no sign of capsular material, while isolates 38W-HC10-1 and 8W-2 showed quantities of an extracellular material covering the cell surface, even after washing. In addition, their surfaces tended to stain unevenly, showing microdroplets of stain, and possibly indicating a hydrophobic surface. All three isolates showed the convoluted surface that is typical of Gram negative bacteria, with no apparent appendages. The

Table 2.4 Cell morphology and motility of several isolates.

Isolate	Gram Stain	KOH Test[1]	Motility[2]
8W-2	Gram -ve rods often in pairs with occasional filamentous forms	-	non-motile
8W-2con	Gram +ve rounded short rods in pairs	+	non-motile
8W-5	Gram variable short fat rods with spindle ends	ND	motile
20S-8	Gram -ve short small rods	-	motile
38W-0-1	Gram variable rods	+	non-motile
38W-0-5	Gram -ve coccobacilli often in pairs	-	non-motile
38W-HC10-1	Gram -ve long thin bacilli often in pairs	-	non-motile
38W-HC10-3	Gram variable spindle-shaped rods	-	non-motile
SL-HC10-1	Gram -ve blunt short bacilli	-	motile
SL-HC10-2	Gram -ve fat short rods	-	motile
SL-HC10-3	Gram variable small spindle-shaped rods often in pairs	+	tumbling

[1] 3% KOH, as described by Von Graevenitz and Bucher (1983):
- = Gram negative; + = Gram positive; ND = Not Determined

[2] determined by hanging drop method (Doetsch, 1981)

reference *P. putida* (ATTC 33015) culture showed polar flagella, no capsular material, but the same uneven surface staining.

Isolates 8W-2 and 38W-HC10-1 grown in ASW + PBa (± supplementation with 5% STCS) usually produced macroscopic strands of extracellular material, which settled out upon standing. When the strands were in the culture medium, they spread out with a rhizoid appearance, complete with filaments and globules of brown material which imparted a brown color to the strands. When the strands were lifted out of the medium they collapsed on themselves and were refractory to physical manipulations such as teasing, maceration and spreading. The strands were very effective in associating with the oil or PBa fraction, and protected the hydrocarbons from solvent extraction; actual physical disruption of the strands was necessary to release the hydrocarbons into the solvent. The close association between the hydrocarbons and the strands also was visible microscopically. When strands were removed from the medium, rinsed with water to remove the growth medium, stained with dilute crystal violet, squashed under a cover slip and observed with oil immersion, they appeared as large purple fibres enclosing non-staining areas of hydrocarbon. Free-floating bacteria were observed in these preparations, but there was no apparent association of bacteria with either the fibres or the hydrocarbons which they enclosed.

Further examination of the strands produced by isolate 8W-2 was performed in a scanning electron microscope to determine whether they were actually cell-free. Scanning electron micrographs (Plate 2.1) show that hydrocarbons were associated with the strands, but in no case were any bacterial cells observed. The strands had a ropy texture which was accentuated by dehydration and solvent washing. Analysis of uncoated fixed strands by X-ray scanning did not reveal any unusual elements, only biological components.

Therefore, although the strands were produced in quantity (usually in a poor medium with hydrocarbons as the major carbon source) and appeared to be hydrophobic, associating intimately with hydrocarbons in the medium, they were apparently cell-free. This precludes the theory that the cells extrude the hydrophobic/lipophilic material as a mechanism for entrapping hydrocarbons to bring them into close contact for metabolism. The function of the strands is unknown, and it is not clear whether they represent the sloughed capsular material observed in the TEM. However, isolate 8W-2 variants have been found which lack both the capsular material and the strands. McMeekin *et al.* (1971) reported the presence of "slime layers" on solid cultures of some Gram negative yellow pigmented bacteria, and noted that these slime layers were cell free. These slime layers may represent material similar to the strands found in cultures of isolates 8W-2 and 38W-HC10-1.

Plate 2.1 Scanning electron micrographs of oil-associated hydrophobic strands from cultures of isolate 8W-2.

Plate A: Fixed material from a culture of isolate 8W-2, washed extensively with methylene chloride to remove all associated hydrocarbons.

Plate B: Fixed material from a culture of isolate 8W-2, washed with a minimal volume of methylene chloride. HC= associated hydrocarbons

POOR COPY
COPIE DE QUALITEE INFERIEURE

A

B

The yellow pigments of isolate 8W-2 were studied spectrophotometrically. According to Reichenbach *et al*. (1980), identification of pigments may be useful in taxonomic classification of Gram negative yellow pigmented rods. Flexirubins are found in a variety of *Cytophaga*-like bacteria, and, unlike carotenoids which may also be present, are found in the outer membrane of the cell wall. These pigments are solvent-extractable without cell lysis, and show a reversible alkali-mediated bathochromic shift in absorption maxima. To determine the character of the yellow pigments in isolate 8W-2, an ethanol extraction of whole cells was spectrophotometrically scanned in ethanol and in 0.1M ethanolic KOH (Weeks, 1980b) (Figure 2.1). The absorption spectrum of the extract was more complex than that reported for flexirubin, and did not show a bathochromic shift in alkali. The spectrum was very similar to that of the carotenoid "Type 2" pigment described by McMeekin *et al*. (1971) and demonstrated a shift of absorption maxima in chloroform identical to the values given by McMeekin *et al*. (1971). (The pigments could not be extracted into chloroform directly, because the cell material formed a stable emulsion when vortexed with $CHCl_3$. Even after 5 d at 22°C, this emulsion was still intact. The reason for or mechanism of this emulsion formation is not known, but may be related to the hydrophobic strands previously observed. It is particularly puzzling that extraction of these putative carotenoid pigments, which are

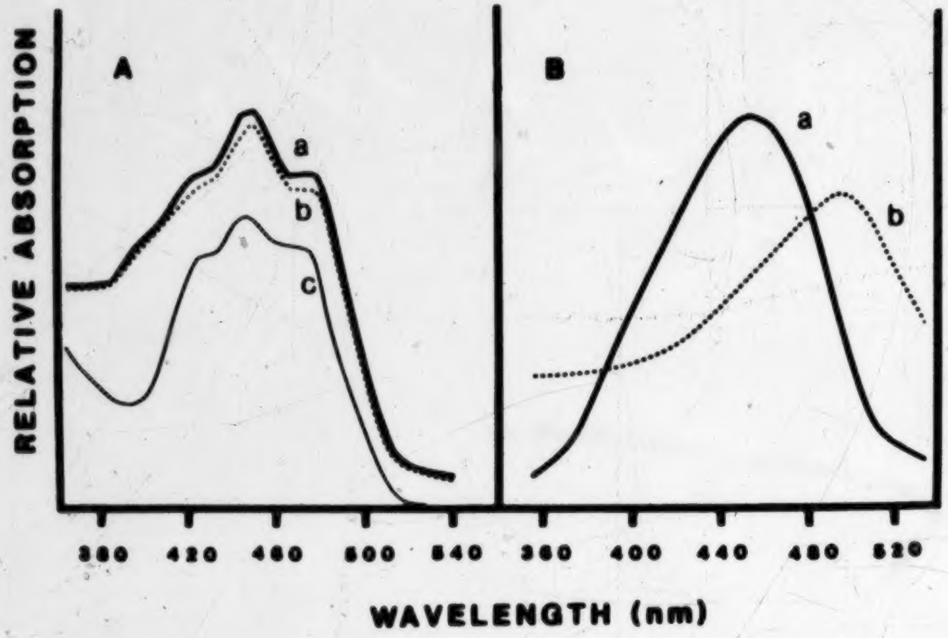

Figure 2.1 Relative absorption spectrum of ethanol-extracted yellow pigments from isolate 8W-2 compared with known *Flavobacterium* pigments.

Panel A: (a) ethanol-extracted pigments of isolate 8W-2 in neutral ethanol; (b) ethanol-extracted pigments of isolate 8W-2 in 0.01M ethanolic KOH; (c) "Type II" pigments of *Flavobacterium* sp. in methanol (adapted from McMeekin *et al.*, 1971)

Panel B: ethanol-extracted pigments of *Flavobacterium odoratum* NCTC 11036 (a) in neutral ethanol; (b) in 0.01M ethanolic KOH (adapted from Weeks, 1980b) supposedly in the inner membrane, was performed so easily with ethanol.)

Enzymatic Activities

The results of oxidase and catalase tests are shown in Table 2.5. All isolates tested were catalase positive and most were oxidase positive.

Only isolate 8W-2 was tested for extracellular DN'ase, and it apparently was weakly positive. However, growth of isolate 8W-2 on the DN'ase medium (Difco) containing methyl green was very slow, and may have influenced the results of the test. Isolate 8W-2 was gelatinase, urease and amylase negative.

Biochemical Capabilities

The results from Oxidative/Fermentative (O/F) glucose plates are shown in Table 2.5. Two isolates (8W-2con and 38W-O-1) failed to grow on the O/F medium; two isolates produced alkaline reactions aerobically (38W-HC10-3 and SL-HC10-2) while the remainder used glucose oxidatively, producing acid. Only two isolates (38W-HC10-1 and SL-HC10-1) were capable of any growth anaerobically on glucose, and this growth was poor. The remaining isolates were characterized as aerobes.

Isolate 8W-2 was found to grow in glucose broth, producing acid but no gas, and but was unable to grow in lactose broth. It grew but did not swarm on Anacker and Ordal (1959) medium.

Table 2.5 Oxidase and catalase reactions of isolates, and growth on oxidative/fermentative (O/F) glucose plates. Catalase and oxidase reactions determined by the method of Smibert and Krieg (1981). O/F glucose plates incubated at 24°C for 48 hr or 7 d.

			O/F Glucose Plates[1]	
Isolate	Oxidase	Catalase	aerobic (48 hr)	anaerobic (7 d.)
8W-2	+	+	+, Acid	-
8W-2con	-	+	-	-
8W-5	+	+	+, Acid	-
20S-8	-	+	+, Acid	-
38W-O-1	-	+	-	-
38W-O-5	+	+	+, Acid	-
38W-HC10-1	+	+	+, Acid	±
38W-HC10-3	+	+	+, Alk	-
SL-HC10-1	+	+	+, Acid	±
SL-HC10-2	+	+	+, Alk	-
SL-HC10-3	-	+	+, Acid	-

---

[1] + = growth; ± = poor growth; - = no growth; Acid=acid produced; Alk=alkaline growth

Antibiotic and metal resistance patterns are given in Table 2.6. Each isolate, including the reference *P. putida* (ATCC 33015), demonstrated resistance to one or more antibiotics. Only mercury and cadmium resistances are presented, since both vanadium and molybdenum results were ambiguous, producing altered colony morphologies.

Temperature limitation studies were not carried out by design. However, the use of high temperatures for curing (Chapter 4) allowed the estimation of growth temperature ranges. Isolate 38W-HC10-1 was capable of growth at 30 and 37°C but grew slowly at 44 and 44.5°C. Isolate 8W-2 would grow at 28 and 30°C but failed to grow at 37°C. The upper limits of growth temperature were not determined for 8W-1, or the other isolates. Minimum growth temperatures were not tested, but all 3 isolates of interest were capable of sustained growth at least at 8°C, since they were originaly isolated at this temperature. It would appear, without conclusive tests, that isolate 38W-HC10-1 is mesophilic while isolate 8W-2 is psychrotrophic.

Formation of endospores was not examined with any isolates, especially since the 3 isolates of particular interest were aerobic or facultative Gram negative rods.

Although isolates 8W-1, 38W-HC10-1 and 8W-2 were originally isolated from marine water samples, none of the isolates demonstrated an absolute requirement for NaCl. Each could grow in non-salty medium, and 8W-2 could tolerate 5.5% NaCl, although the upper limits of tolerance were not

Table 2.6 Antibiotic and metal resistance of isolates, as determined on solid media.

Isolate	Antibiotic Discs[1]								Metals[2]	
	Am	Cb	Gm	K	N	Nb	Sm	Te	Hg	Cd
8W-1	R[3]	R	S	S	S	R*	R*	S	S	S
8W-2	S	S	S	S	S	S	R	S	R	R
8W-2con	R*	S	R	R	R	S	R	R	-	-
8W-5	S	S	R	R	R*	-	R	R*	-	-
20S-8	S	S	S	S	S	R*	S	R	S	S
38W-O-1	S	S	R	R	S	S	R	R	-	-
38W-O-5	S	S	S	S	S	-	S	R	-	-
38W-HC10-1	S	S	S	R*	S	R	S	R	R	S
38W-HC10-3	S	S	R*	S	S	S	S	R	-	-
SL-HC10-1	R	R	R*	S	R*	R	S	S	-	-
SL-HC10-2	R*	S	S	S	S	S	S	S	-	-
SL-HC10-3	S	S	R*	R*	R*	S	S	R*	-	-
P.putida	R	R	R*	S	R*	R	S	S	S	R

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[1] Antibiotics: Am = Ampicillin, 10µg; Cb = Carbenicillin, 100µg; Gm = Gentamicin, 10µg; K = Kanamycin, 30µg; N = Neomycin, 30µg; Nb = Novobiocin, 300µg; Sm = Streptomycin, 10µg; Te = Tetracycline, 30µg

[2] Metals: Hg = 10µg $HgCl_2$/mL; Cd = 100µg $CdCl_2$/mL

[3] R = resistant; R* = tentatively resistant; S = sensitive; - = not determined (see text)

determined.

The results of these tests show that there is taxonomic diversity in the isolates capable of oil degradation. Comparison of characteristics of the isolates with Bergey's manual (Buchanan and Gibbons, 1974) (using a computer-assisted taxonomic scheme based on Bergey's manual to aid in preliminary identification) resulted in the tentative identifications shown in Table 2.7. Some isolates could not be assigned to genera on the basis of the limited tests performed. The 38W-HC10-1 isolate was tentatively identified as an *Aeromonas* sp. based on the following criteria: Gram negative rod; oxidative and weakly fermentative reaction on glucose O/F plates; positive oxidase and catalase tests; lack of motility; and lack of soluble pigment from colonies. Isolate 8W-1, which was originally identified as a *Flavobacterium* sp. by Clare[3], was reclassified by Fedorak and Westlake (1983) as an *Acinetobacter* sp. Of the three isolates chosen for genetic work (8W-1, 8W-2 and 38W-HC10-1), the identification of isolate 8W-2 as a *Flavobacterium* is the most uncertain. This is mainly because of the poor definition of *Flavobacterium* as a genus.

A Discussion of Flavobacterium Taxonomy

The positive identification of isolate 8W-2 as a *Flavobacterium* was important in terms of finding literature

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[3] Clare,R. 1981. Microbial degradation of Prudhoe Bay crude oil by isolated species of marine bacteria. 65pp. Project Report for Microbiology 495. Dept. Microbiology, University of Alberta.

Table 2.7 Tentative generic identification of isolates.

Isolate	Identification	Reference
8W-1	*Flavobacterium* *Acinetobacter*	Clare[1] Fedorak and Westlake (1983)
8W-2	*Flavobacterium*	Clare[1], this study
8W-2con	*Bacillus*	this study
8W-5	*Bacillus*	Clare[1], this study
20S-8	*Pseudomonas*	Clare[1], this study
38W-O-1	*Bacillus*	this study
38W-O-5	unknown	this study
38W-HC10-1	*Aeromonas*	this study
38W-HC10-3	unknown	this study
SL-HC10-1	*Pseudomonas*	this study
SL-HC10-2	unknown	this study
SL-HC10-3	unknown	this study

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[1] Clare, R. 1981. Microbial degradation of Prudhoe Bay crude oil by isolated species of marine bacteria. 65pp. Project report for Microbiology 495, Department of Microbiology, University of Alberta.

references for genetics of the genus. Establishment of a plasmid-bearing hydrocarbon-degrading *Flavobacterium* was of importance, since there are very few literature references to flavobacteria with plasmids, degradative or otherwise.

There is ample evidence for a close relationship between *Flavobacterium* and *Cytophaga* (Shewan and McMeekin, 1983). The taxonomy of the Flavobacterium/Cytophaga group is very vague and misleading, in part because the genus *Flavobacterium* has become a "dumping ground" for poorly defined species (Weeks, 1980a). Even the type species of the genus, *Flavobacterium aquatile*, has come under criticism as being unrepresentative of the majority of *Flavobacterium* isolates (Shewan and McMeekin, 1983; Thomson and McMeekin, 1980; Weeks, 1980a;b; Holmes and Owen, 1980).

There are many taxonomic schemes for differentiating putative flavobacteria into groups, but few of the comparative studies have been done with comparable isolates. Rather, it becomes clear from the literature that a number of taxonomic tests are necessary to divide the flavobacteria into groups and to separate them from the *Flexibacter* and *Cytophaga* genera. In many cases, the isolates cannot be speciated, and there is much crossover between groups with many exceptions to the groups.

One significant test appears to be the determination of G+C% content of chromosomal DNA. Researchers seem to agree that there are two natural groupings of presumptive flavobacteria: the non-motile species with low G+C% contents

(30-45%) and those species with high G+C% contents (53-69%). It has been suggested that the low G+C% species be retained as *Flavobacteria* while the high G+C% species be accomodated in a new genus (Hayes and Mapp, 1980). The inclusion of G+C% content determinations for isolate BW-2 would have been helpful in assigning it to a genus.

Another potentially useful taxonomic aid is the identification of cell pigments, but their classification is far from complete. Flexirubin pigments are found in most strains of *Flexibacter*, and *Cytophaga*-like bacteria from soil and freshwater, and from most low G+C% flavobacteria, but not in those of marine origin (Reichenbach *et al.*, 1980). As well, recent reports (Mullings and Parish, 1980) that some *Cytophaga*-like bacteria isolated from terrestrial and freshwater samples gave negative spectrophotometric results for flexirubin, cast doubt upon the significance of flexirubins in the taxonomy of the *Flavobacterium/Cytophaga* group. However, Reichenbach (personal communication cited by Shewan and McMeekin, 1983) has found that the presence of flexirubins may be obscured by the presence of carotenoids. McMeekin *et al.* (1971) also studied the pigments of Gram negative yellow pigmented rods, but were unable to assign any taxonomic significance to the carotenoid pigments that they described, since groupings based on pigments did not completely correlate with groupings based on biochemical characteristics. Gandy and McMeekin (cited by Shewan and McMeekin, 1983) found that a number of low G+C% marine flavobacteria contained extractable pigments similar to the Type 2 pigment described by McMeekin *et al.* (1971). Therefore, finding presumptive carotenoid Type 2 pigments in isolate 8W-2 does not preclude the presence of flexirubins, and even if flexirubins were not present, this would not prevent 8W-2 from being classified as a marine *Flavobacterium*.

The presence of a slime layer (i.e. the material observed by scanning electron microscopy) or excessive capsular material (observed by transmission electron microscopy) does not aid in classifying 8W-2, since McMeekin *et al.* (1971) found that slime layers were not restricted to either *Cytophaga* or *Flavobacterium* species.

A complication of taxonomic division is that certain flavobacteria are found in certain environments. For example, of 94 presumptive high G+C% flavobacteria, 54 which were oxidase positive and peritrichously flagellated were isolated from sewage sludge, water, milk, or leaf surfaces, while 29 which were oxidase negative and non-motile were isolated from poultry, pork or lamb raw meats (Hayes and Mapp, 1980). Thus, the marine origin of 8W-2 may be significant, as noted by the following quotation from Hayes (1977) regarding flavobacterium taxonomy:

> "...it is worth noting that isolates from the marine environment have phenetic characters which markedly differ from the terrestrial isolates studied here".

Hayes (1977) suggests that a new description of *Flavobacterium* be accepted into Bergey's manual, namely:
Gram negative rods to near coccal forms, often developing as longer rods in liquid media. Non-motile, do not glide on solid media. Do not form endospores. Typical colonies pigmented orange-yellow, yellow, or greenish-yellow, convex, circular, smooth, shiny, entire, translucent. Growth range 30 to 0°C, heat labile. Often proteolytic, usually dissimilate glucose oxidatively with production of acid only. May also attack maltose, starch and sucrose. Essentially obligate aerobes. Catalase, oxidase and phosphatase positive. Usually all IMViC tests negative, urease negative. Require supplementary growth factors. Widely distributed, found on raw meats, in raw milk, soil, and fresh and marine waters. DNA base ratios (G+C% content) 30 to 42%.

The isolate 8W-2 would fit nicely into this revised description of *Flavobacterium* based on those taxonomic tests which have been carried out. Some tests have not been performed, namely endospore formation, heat lability, phosphatase and IMViC tests, and determination of G+C% content. Contrary to Hayes' description, isolate 8W-2 does not seem to require growth factors, but certainly grows much better in basal salts media when supplemented with 0.01% YE or 0.5% TCS.

Thus, isolate 8W-2 should be classified as a member of the *Flavobacterium* genus. It fits better into the proposed revised taxonomy schemes than it does into extant definitions in Bergey's manual (8th Ed.), which have been subject to considerable criticism.

If it is a flavobacterium, then this is one of very few published reports of putative degradative plasmids in a *Flavobacterium* species. The only other reports that I am aware of are those of Negoro *et al*. (1980; 1982; 1983; 1984) based on *Flavobacterium* sp.KI72, an isolate which was originally classified as *Achromobacter guttus* . The basis for this strain's reclassification to *Flavobacterium* sp. was not presented in those reports.

2.3.3 Increasing Cell Yield

It was known that *Acinetobacter* (8W-1) and *Flavobacterium* (8W-2) could grow on whole PB crude oil, since they were maintained by monthly transfer at 8°C with PB crude oil as sole carbon source. Also, it was known that they could alter the GC profile of the aromatic fraction, since the isolates were chosen for this ability, but it was not known whether *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) or *Aeromonas* (38W-HC10-1) could actually grow on PB aromatics. A time course study of the three isolates incubated with PBa as sole carbon source is shown in Figure 2.2. The graph shows that growth occured in all three cultures. An initial growth period in the first week after transfer was followed by a slow decline in viable numbers. Isolate *Aeromonas* (38W-HC10-1) grew to a higher cell density than

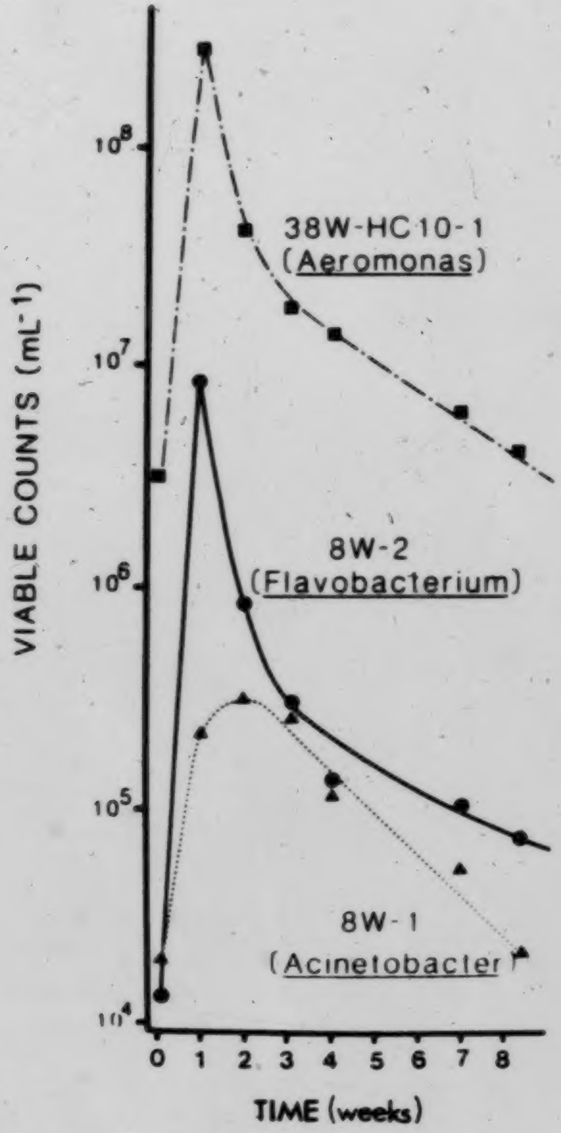


Figure 2.2 Growth of *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) maintenance cultures with PB aromatics. Graph of viable numbers versus incubation time, determined by plate count. Each point represents the mean of 3 values. Cultures were grown in ASW + NP with 0.1%v/v PBa as sole carbon source at 26°C.

Acinetobacter (8W-1) or *Flavobacterium* (8W-2), yet *Flavobacterium* (8W-2) showed the largest initial increase in viable numbers, increasing by over 600-fold. Isolate *Aeromonas* (38W-HC10-1) increased by approximately 100-fold, and *Acinetobacter* (8W-1) by only 11-fold. These increases seem to reflect the degree of aromatic degradation displayed by these cultures in screening studies (Table 2.2; Section 2.3.1). That is, *Flavobacterium* (8W-2) shows removal of the largest number of aromatic peaks from the GC profile, while *Aeromonas* (38W-HC10-1) is more limited in degradative power, and *Acinetobacter* (8W-1) is the most limited, using only biphenyls; the cultures are growth-limited by the amount of "usable" substrate in the aromatic fraction, and this differs for each isolate.

Because of the limited "usable" carbon available in PBa, cell yields from maintenance medium (i.e. ASW + NP + PBa) were low and growth rates were very slow for all 3 isolates. For plasmid work, it was necessary that cell yields be increased and that the incubation time be reduced from weeks to days. However, it was also thought necessary that the medium employed provide a selective pressure for degradative plasmids, preventing the use of rich media. Initial attempts to optimize cell yields using 1L volumes of minimal medium (ASW + NP + 0.01% YE plus dibenzothiophene, phenanthrene or naphthalene) resulted in an increase in turbidity within 3 d, probably due to growth on the 0.01 to 0.05% YE supplement. Metabolism of dibenzothiophene and phenanthrene was inferred from the orange color produced in the media within 3 d of inoculation. No color and little turbidity were observed in the naphthalene flask. The cell pellets obtained after 2 weeks incubation were very small, and all attempts to remove contaminating PAH from the pellet by solvent extraction resulted in lysis of the cells. This method was obviously inadequate for providing sufficient cell material in a short time.

None of the supplemented media provided a quick high yield of cells, although carbon source experiments did show an increase in cell yield proportional to the supplement. Contrary to the findings of Aranha and Brown (1981), the source of nitrogen in the medium made no difference to the degree of aromatics degradation by *Flavobacterium* (8W-2), and apparently did not increase growth. However, the presence of 0.01% to 0.05% w/v yeast extract definitely enhanced growth as noted by increased culture turbidity. Similarly, the addition of small amounts of succinate, as proposed by Finnerty *et al.* (1983), or sodium acetate slightly increased cell yield without stimulating degradation. Hou and Laskin (1976) and Dibble and Bartha (1979) suggested that ferrous iron may be a limiting factor in PAH degradation, but inclusion of trace iron in the medium did not result in increased cell yield in this study. This suggested that, in a minimal medium + NP with PAH as sole carbon source, the growth-limiting factor was a readily utilizable carbon source.

Hou and Laskin (1976) suggested that the use of hydrophobic solvents such as mineral oil or surfactants such as Tween 80 was stimulatory to the degradation of very insoluble PAH such as dibenzothiophene, probably by presenting the substrate in a form more available for microbial attack. In an attempt to increase the amount of available PAH for cell growth, supplementation of *Flavobacterium* (8W-2) cultures with mineral oil, Tween 80 and combinations of these supplements with iron was tried. There was no significant increase in cell yield (the mineral oil did not serve as a carbon source since it is composed of paraffins, which *Flavobacterium* (8W-2) cannot degrade). This again indicated that the growth was limited by available carbon, and that the aromatic fraction was too poor a carbon source to allow extensive growth. For this reason, minimal media ± supplements were abandoned in favor of media with a rich carbon source to provide a quick yield of cell material.

Good growth was observed in flasks with rich media (e.g. STCS plus PB or PBa), as evidenced by the cultures' turbidity. However, extraction of the residual oil or aromatic fraction for GC assay was hindered by emulsification of the oil in the complex medium. Both acid and alkaline re-extractions of the medium after neutral extractions with methylene chloride were necessary to recover the residual hydrocarbons. Even so, some low molecular weight hydrocarbons were not recovered, even from uninoculated medium. New spurious peaks were found in gas chromatograms from extracts of sterile medium, and more peaks apeared in spent medium. These peaks interfered with degradation analysis. As well, the emulsified oil from inoculated medium made pelleting of cells difficult and interfered with plasmid preparation procedures.

Eventually, it was decided that the two problems be separated and treated differently. To ensure constant selective pressure, maintenance cultures were set up in minimal medium with PBa as sole carbon source. These media (as well as BMA plates inoculated from the cultures) were used as sources of inoculum for rich medium flasks (eg. dilute STCS or full strength STCS) to produce large cell pellets. These rich media were incubated a minimum amount of time (usually 20 to 48 hours, only until turbid) to minimize the exposure of the isolates to non-hydrocarbon medium. In this manner, it was hoped that selective pressure could be maintained on the inoculum, while sufficient quantities of cells would be quickly produced for plasmid preparations. (PAH degradation experiments, however, were designed with minimal medium because of the interference produced by STCS.) Re-inoculation of minimal medium + PBa with 5% v/v STCS-grown culture resulted in aromatic degradation, demonstrating that the degradative capability of the culture is not lost after one transfer in STCS.

The growth of *Flavobacterium* (8W-2) in fermenter jars using rich medium (STCS) is shown in Figure 2.3. The frozen

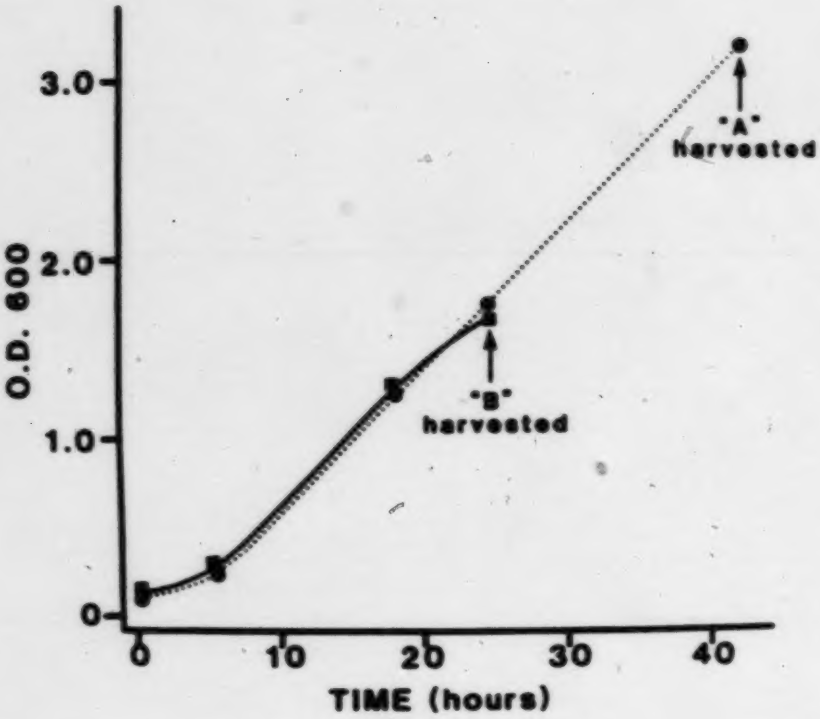


Figure 2.3 Growth of *Flavobacterium* (8W-2) in large scale cultures of STCS medium. Graph of culture optical density at 600nm versus incubation time for 10.5L cultures grown in fermenter vessels. Incubation ocurred at 26°C with 4L air per min and 100 rpm stirring.

cell paste from these jars was used as sources of cell material for plasmids and cell wall pigments in taxonomy.

2.3.4 Degradative Capabilities of *Acinetobacter* (8W-1)

Degradative capabilites of *Acinetobacter* (8W-1), *Aeromonas* (38W-HC10-1) and *Flavobacterium* (8W-2) were known from GC results to be different (Table 2.2). Growth of these isolates on various PAH was studied individually, using plate counts, mineralization of labelled compounds and autoradiography.

Isolate *Acinetobacter* (8W-1) was known to be limited to degradation of biphenyl and methylbiphenyls (Fedorak and Westlake, 1983). It was found to be capable of mineralization of (U-^{14}C)-biphenyl, and this metabolism was influenced by the growth medium (Table 2.8). The greatest amount of growth (i.e. turbidity) occurred in flasks containing the rich medium, STCS, yet the greatest amount of mineralization of ^{14}C-biphenyl occurred in the flasks containing basal medium and unlabelled biphenyl. This might indicate induction of biphenyl degradation in *Acinetobacter* (8W-1), independent of growth rate. Volatility controls were similar, independent of the culture medium.

2.3.5 Degradative Capabilities of *Aeromonas* (38W-HC10-1)

When the same type of experiment was carried out with *Aeromonas* (38W-HC10-1) and phenanthrene, it was found that the isolate was incapable of releasing ^{14}CO$_2$ from (9-^{14}C)-

Table 2.8 Mineralization of (U-^{14}C)-biphenyl by *Acinetobacter* (8W-1) in 4 media. Cultures were incubated for 16 d at 22°C in the dark, with shaking.

	% of Label Trapped		
Medium[1]	Test	Control[2]	Net[3]
Basal + 50µg biphenyl	31.7	8.7	23.0
Basal + 50µL PB	31.8	9.9	21.9
Basal + 50µL PBa	19.0	8.6	10.4
STCS	9.8	6.4	3.4

[1] Each flask spiked with ≈ 7,000 dpm of ^{14}C-biphenyl
Basal medium = ASW + NP + 0.01% yeast extract

[2] Uninoculated volatility controls

[3] Net = % label released as $^{14}CO_2$ (Test - Control)

phenanthrene. This observation should not be explained by lack of induction, because the media contained phenanthrene, PB oil or PBa, any of which should induce phenanthrene degradation. Other experiments (data not reported) confirmed this observation. However, the isolate did alter phenanthrene, as indicated by loss of its corresponding peak from the GC profile (Appendix). To resolve this apparent conflict, thin-layer chromatography and autoradiography were employed.

On the premise that initial oxidative attack on hydrocarbons produces compounds with increased polarity, a thin-layer chromatography (TLC) system was developed for separating non-polar parent compounds from polar oxidation products. Isolate *Aeromonas* (38W-HC10-1) was incubated with unlabelled phenanthrene or with phenanthrene in PBa. When solvent extracts of these cultures were spotted on TLC plates, developed in non-polar solvents, and observed under ultraviolet light, new fluorescent areas appeared. These new compounds had not migrated far from the origin, which, under the chromatography conditions used, was indicative of polar properties. In order to prove that these new compounds were derived from phenanthrene, rather than other medium components, it was necessary to employ ^{14}C-labelled phenanthrene and use autoradiography to determine the fate of the radioactive substrate. In all cases, uninoculated flasks containing radioactive substrate were used as negative controls. The media chosen for the experiment were designed to stimulate phenanthrene-degradative activity, in much the same way as the media used to stimulate *Acinetobacter* (8W-1) to degrade ^{14}C-biphenyl. Culture extractions with $CHCl_3$ were tested under basic, neutral and acidic conditions; the acidic extraction conditions showed the largest number of new compounds (i.e. those migrating with polar reference compounds rather than with the parent compounds). Extracts of parallel inoculated cultures which did not contain phenanthrene did not show these new compounds, indicating that the extra spots were peculiar to phenanthrene-containing cultures, and that the acidic conditions were not merely extracting new compounds from unaltered culture medium. Therefore, acidic extraction conditions were routinely used. Liquid scintillation counting of the aqueous phase left after extraction indicated that the majority of the label was extracted under these conditions. The solvent systems employed were able to separate naphthalene and phenanthrene from 2-naphthol and phenol. Extracts of cultures containing PBa showed long trailing smears of fluorescent material on TLC due to the heterogeneous nature of the PBa, but this material had no effect on autoradiography results. Radioactive spots were seen near the TLC origin from 7 day culture extracts. The intensity of these radioactive regions increased with incubation time of the cultures. A schematic representation of the autoradiogram from 21 day cultures is shown in Figure 2.4. The uppermost spots represent unaltered ^{14}C-

Figure 2.4 Representation of autoradiogram produced by TLC of 21 day culture extract of *Aeromonas* (38W-HC10-1) incubated with (9-^{14}C)-phenanthrene-spiked PBa or phenanthrene.

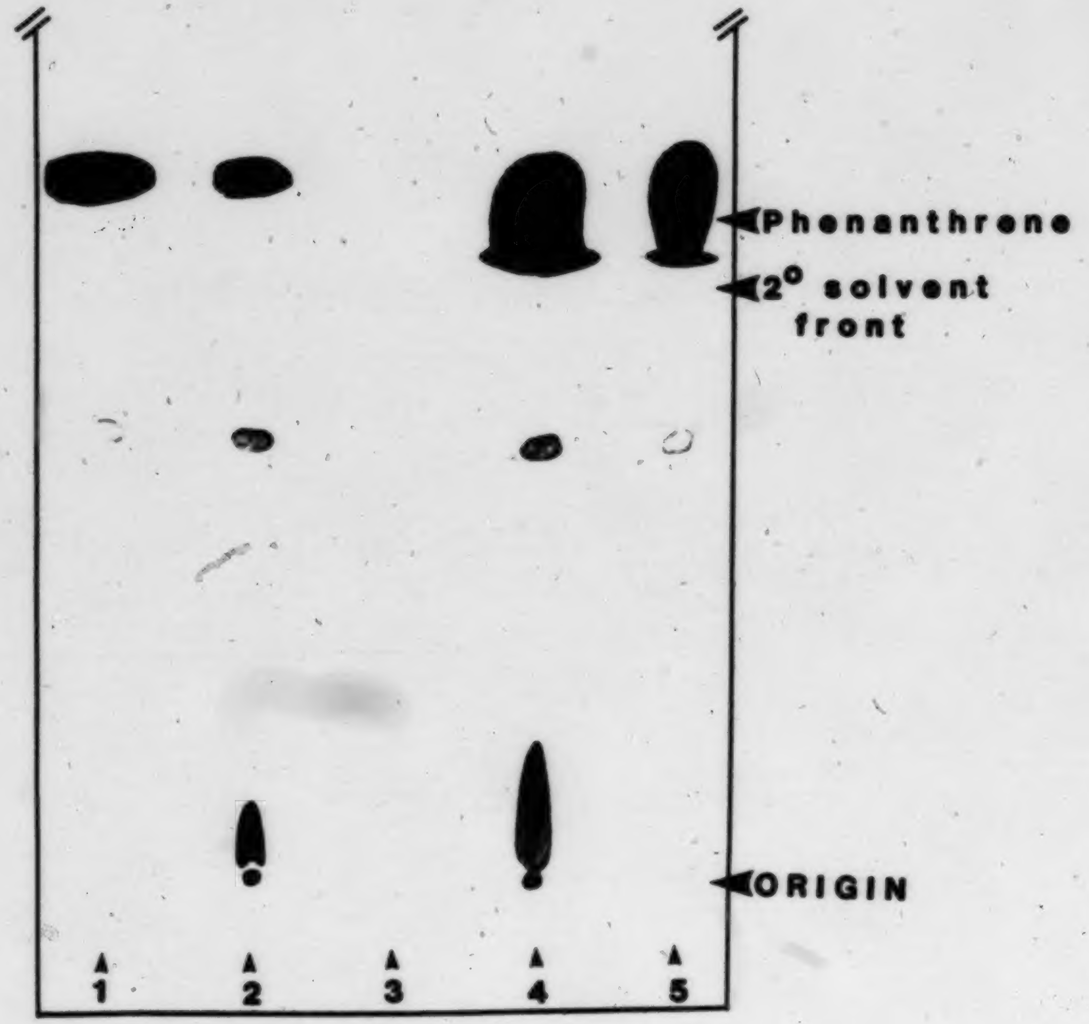
Lane 1: Uninoculated control incubated with (9-^{14}C)-phenanthrene-spiked PBa;

Lane 2: Inoculated test culture incubated with (9-^{14}C)-phenanthrene-spiked PBa;

Lane 3: Non-radioactive authentic phenanthrene (U.V. marker);

Lane 4: Uninoculated control incubated with (9-^{14}C)-phenanthrene-spiked phenanthrene;

Lane 5: Inoculated test culture incubated with (9-^{14}C)-phenanthrene-spiked phenanthrene.


Phenanthrene
2° solvent front
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phenanthrene, as determined by co-migration with authentic phenanthrene (U.V. visualization). In the original autoradiogram, there are faint spots visible immediately below the authentic phenanthrene. These spots are present in all 4 culture lanes in approximately the same relative intensity, whether from test or control cultures, and may represent a radioactive impurity in the (9-^{14}C)-phenanthrene or a spontaneous oxidation product. At and near the origin of both test lanes are found smears of radioactive material. These spots are absent in uninoculated controls, and are interpreted to be partially metabolized (9-^{14}C)-phenanthrene. There is relatively more of the radioactive material in the test lane from phenanthrene-amended medium than from the PBa-amended medium. Whether or not this implies induction of partial oxidation in the presence of phenanthrene cannot be decided in this experiment.

It is apparent that *Aeromonas* (38W-HC10-1) is capable of only limited phenanthrene degradation. The isolate does not mineralize the compound, but alters it sufficiently to remove its corresponding peak from GC profiles and to produce polar compounds, as indicated by TLC. This cooxidation of phenanthrene is not capable of supporting growth of *Aeromonas* (38W-HC10-1) on phenanthrene as sole carbon source, as can be seen in Figure 2.5. Comparing growth in the two flasks, it appears that all growth in the phenanthrene-containing flask is due to growth on the yeast extract supplement, since viable numbers parallel the growth

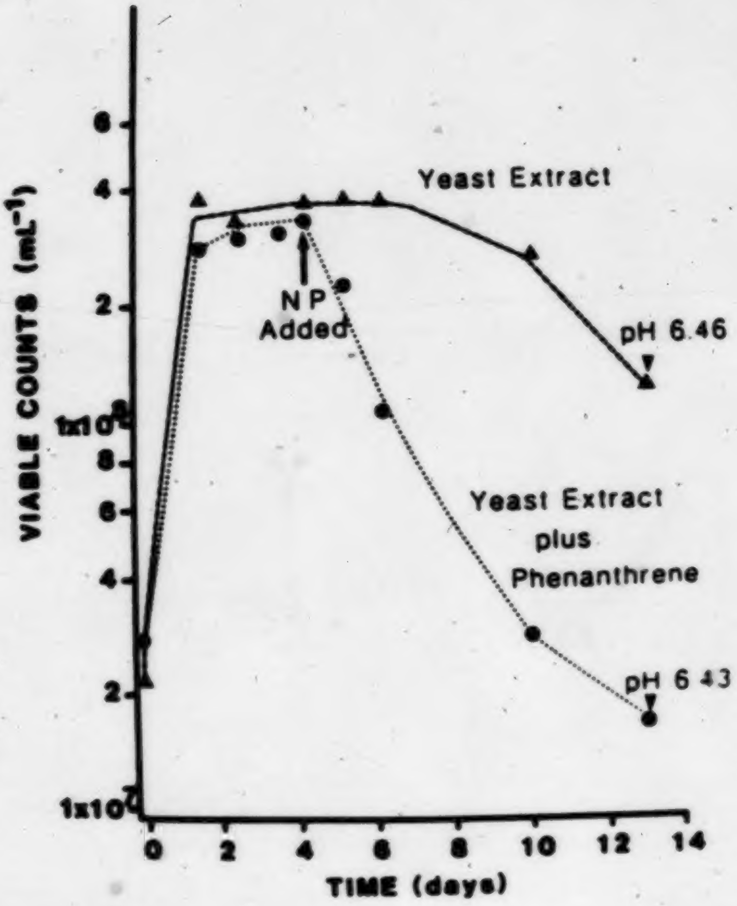

Figure 2.5 Growth of *Aeromonas* (38W-HC10-1) with yeast extract and phenanthrene. Graph of viable numbers versus incubation time in ASW + 0.01% YE ± 5.6mM phenanthrene. Each point represents the mean of 3 plate count values. NP supplement added to each culture 4 days after inoculation.

in the medium containing only yeast extract. When a supplement of nitrogen and phosphorus was added to the phenanthrene-containing medium, the viable numbers decreased. No explanation can be found for this phenomenon of die-off. It is not related to pH differences, since the final pH values were similar.

2.3.6 Degradative Capabilities of *Flavobacterium* (8W-2)

Figures 2.6 and 2.7 demonstrate the ability of *Flavobacterium* (8W-2) to degrade a variety of aromatic compounds within a complex mixture such as crude oil. The capillary gas chromatograms compare an uninoculated PB oil (control) with PB oil which has been incubated with a pure culture of *Flavobacterium* (8W-2). The *Flavobacterium* isolate caused reduction in the peak heights of the methylnaphthalenes (Region 1), dimethylnaphthalenes (Region 2), dibenzothiophene (Peak 3), phenanthrene (Peak 4), the methylphenanthrenes (Region 5) and specific compounds of the C_2-phenanthrenes (Region 6; Figure 2.6). It is also capable of degrading naphthalene and biphenyls (peaks not shown). In the sulfur heterocycle component of the aromatics (Figure 2.7), *Flavobacterium* (8W-2) caused peak height reduction of dibenzothiophene (Peak a), and the methyl-dibenzothiophenes (Peaks b,c and d). It is also capable of attacking the benzo(b)thiophenes (peaks not shown). This reduction in peak heights may represent complete degradation of the compound (mineralization) or simply alteration of the parent molecule

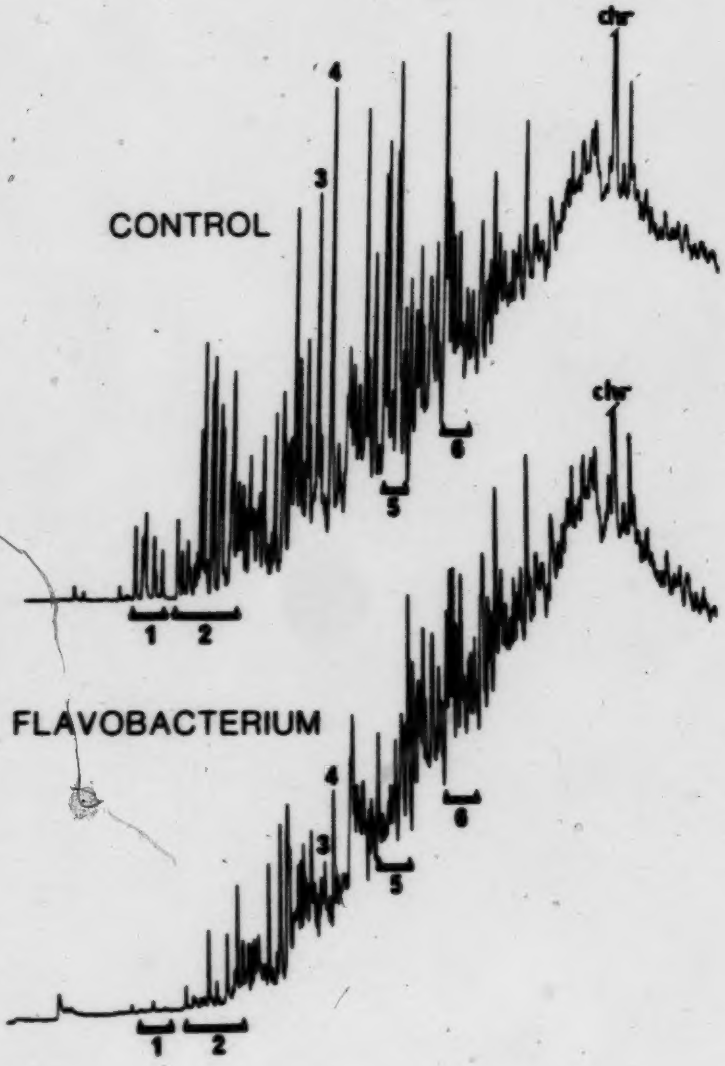

Figure 2.6 Capillary gas chromatograms of the aromatic fraction of PB crude oil incubated with *Flavobacterium* (8W-2) or with uninoculated medium (control). Chromatograms are directly comparable. Peak Identification: Region 1: C_2-Naphthalenes; Region 2:C_3-Naphthalenes; Peak 3: Dibenzothiophene; Peak 4: Phenanthrene; Region 5: Methylphenanthrenes; Region 6: C_2-Phenanthrenes.

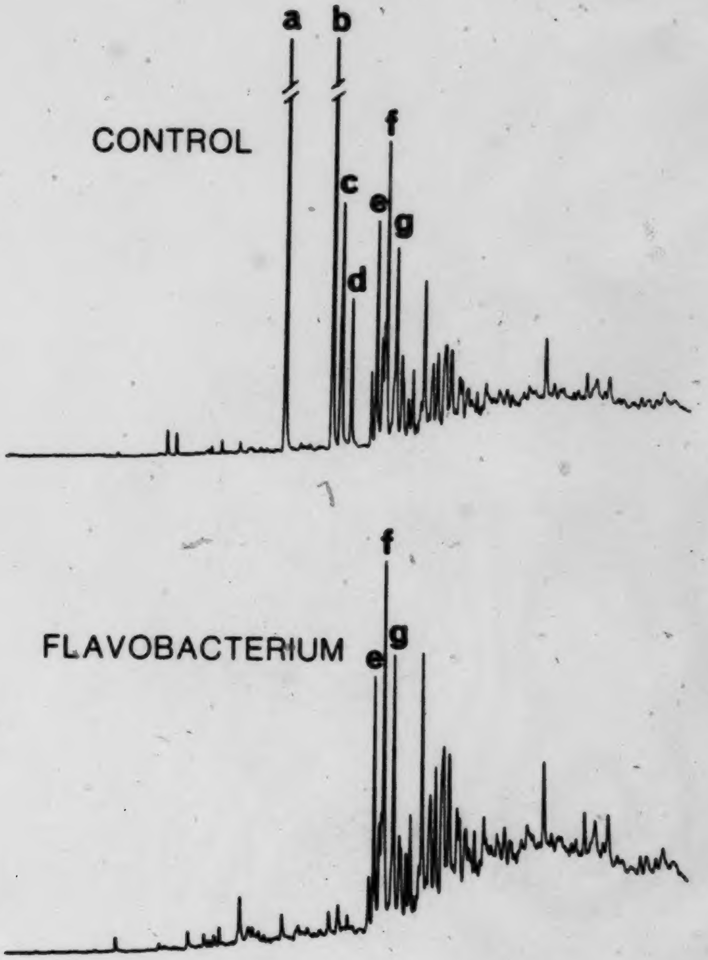

Figure 2.7 Capillary gas chromatograms of the sulfur heterocycles of PB crude oil incubated with *Flavobacterium* (8W-2) or with uninoculated medium (control). Chromatograms are directly comparable. Peak Indentification: Peak a: Dibenzothiophene; Peaks b,c,d: C_1-Dibenzothiophenes; Peaks e,f,g: C_2-Dibenzothiophenes.

sufficient to cause it to adsorb to the column material during chromatography (e.g. through the addition of oxygen molecules).

Growth of *Flavobacterium* (8W-2) on pure single substrates is shown in Figure 2.8, compared with growth on the complex PBa substrate. It can be seen that growth occurred in the presence of PBa and 5mM phenanthrene, but not in the "no carbon" control, nor in the presence of 5mM biphenyl or dibenzothiophene. This would imply that *Flavobacterium* (8W-2) is capable of altering biphenyl and dibenzothiophene (since it removes the corresponding peaks from GC profiles; Figure 2.6) but is incapable of degradation extensive enough to allow growth, similar to the phenomenon of cooxidation previously discussed. Phenanthrene would by inference be more completely metabolized, thereby allowing for growth of *Flavobacterium* (8W-2) with phenanthrene as sole carbon source. This growth should not be a simple reflection of solubility, since the media are saturated with hydrocarbons. If solubility was limiting in the case of biphenyl and dibenzothiophene, slow growth should result rather than culture death parallel to the "no carbon" control. By the same reasoning, toxicity should not be the reason for culture death, since the curve parallels the "no carbon" control. It is probable that only partial oxidation of these substances is occurring, insufficient to allow growth.

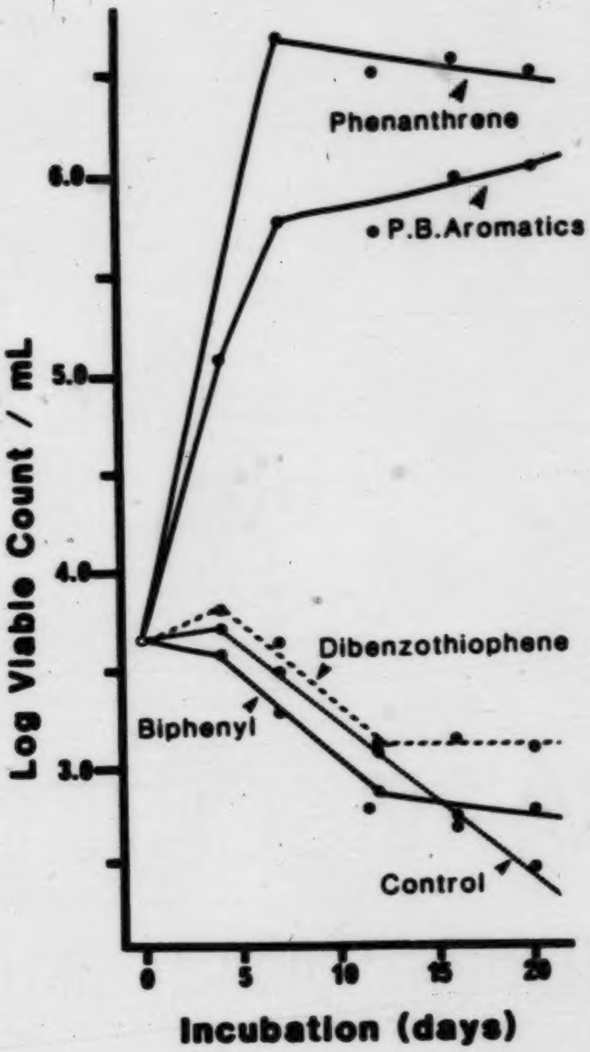

Figure 2.8 Growth of *Flavobacterium* (8W-2) with PBa or pure hydrocarbon substrate as sole carbon source. Graph of viable numbers versus incubation time in ASW + NP ± 50µL PBa or 5mM pure substrate. Each point represents the mean of 3 values. Control medium contains no carbon source.

(9-^{14}C)-Phenanthrene was mineralized quickly by *Flavobacterium* (8W-2), as shown in Figure 2.9. From 25 to 60% of the label was released as $^{14}CO_2$, depending on temperature and culture conditions. There were no apparent induction effects like those seen with *Acinetobacter* (8W-1) and biphenyl mineralization. Because the label is located in the internal position, this release of $^{14}CO_2$ represents extensive metabolism of the compound.

Evidence for the partial oxidation of anthracene, a structural analogue of phenanthrene, was gathered by radiometric means, measuring the release of $^{14}CO_2$. Anthracene labelled with ^{14}C in different positions was used: one set of flasks contained anthracene which was uniformly labelled on the outside ring; one set contained anthracene labelled on the single innermost carbon. The label was added to PBa in ASW + YE. Growth was observed in all test flasks as increasing turbidity. Table 2.9 shows the net % label released as $^{14}CO_2$. The % of label trapped increased in both test and control flasks with time. The net % label released as $^{14}CO_2$ shows that 12% of the side-ring labelled anthracene was sufficiently metabolized to release $^{14}CO_2$, while the internally labelled anthracene did not show liberation of $^{14}CO_2$. This is consistent with the anthracene degradation pathway described by Cripps and Watkinson (1978) where anthracene is subjected to cleavage of the outermost ring. Incomplete metabolism of the ring fission compound produces 2-hydroxy-3-naphthoic acid which contains the

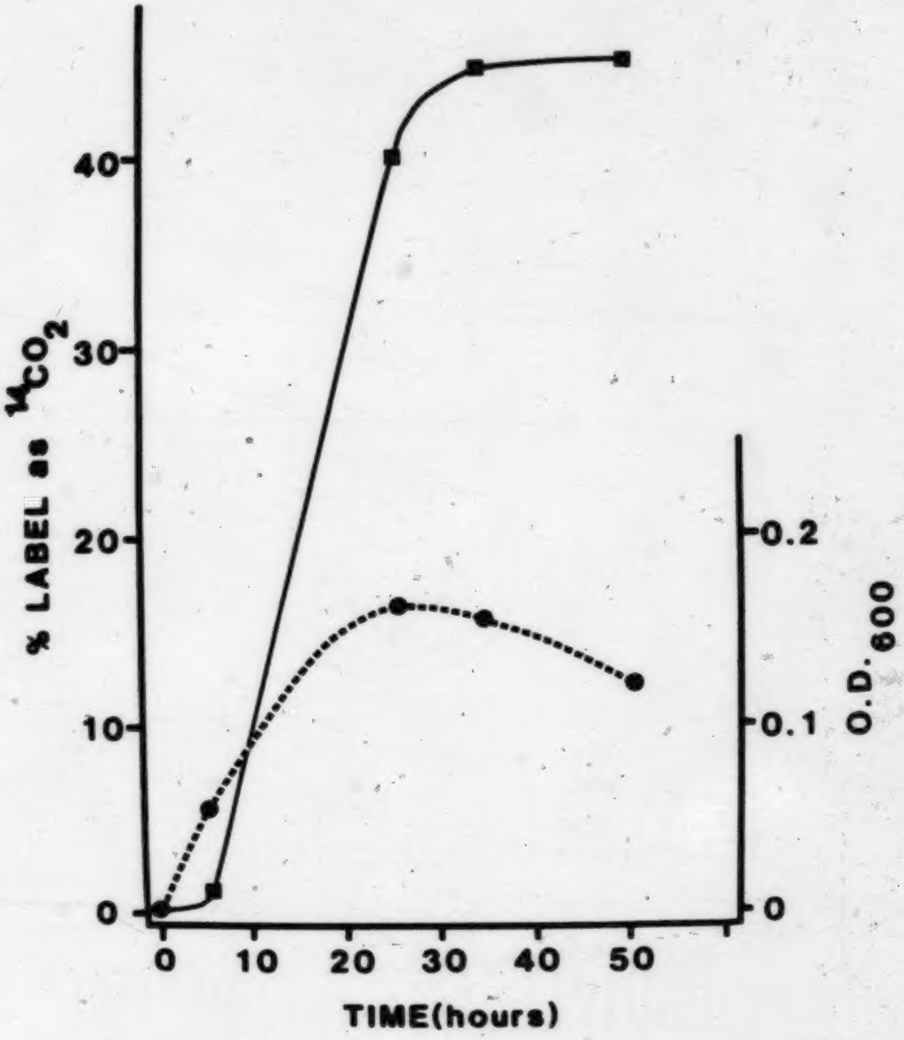

Figure 2.9 Mineralization of (9-^{14}C)-phenanthrene by *Flavobacterium* (8W-2). Graph of $^{14}CO_2$ release (solid line) and growth of *Flavobacterium* (8W-2) measured by culture turbidity (broken line) versus incubation time. Cultures incubated in ASW + 5% TCS with (9-^{14}C)-phenanthrene at 26°C.

Table 2.9 Mineralization of ^{14}C-anthracene by *Flavobacterium* (8W-2). Cultures incubated at 22°C in ASW + 0.05% YE plus 50µL PBa spiked with ^{14}C-anthracene. Control cultures were uninoculated.

	% Label Trapped					
	43 hr			209 hr		
Anthracene label position	Test	Control	Net[1]	Test	Control	Net
Side-(U-^{14}C)	13.4	2.0	11.4	18.7	6.6	12.1
(9-^{14}C)	3.0	3.3	0	4.2	8.2	0

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[1] Net=% label released as $^{14}CO_2$ (Test - Control)

uncleaved dicyclic moiety. The pathway for degradation of this compound is not known, but some organsisms eventually produce salicylate or catechol. It can be concluded that *Flavobacterium* (8W-2) is capable of fission of the outer ring (to release $^{14}CO_2$) but is not capable of continuing metabolism, leaving the remaining two rings intact.

(7,10-^{14}C)-Benzo(a)pyrene and (9-^{14}C)-fluorenone were incubated with *Flavobacterium* (8W-2) to test for mineralization, but no release of $^{14}CO_2$ was observed. This may be a result of the position of the label, as discussed above with anthracene.

Thus, *Flavobacterium* (8W-2) is capable of extensive metabolism of phenanthrene while metabolism of anthracene is incomplete. Although both these compounds disappear from GC traces of the aromatic fraction, the extent of their metabolism cannot be inferred from GC profiles. The use of radiolabelled compounds reveals the different fates of these compounds.

In summary, three marine bacterial isolates were selected which displayed differing PAH-degradative capabilities, and contained plasmids (Chapter 3). Through various taxonomic tests, the three isolates were tentatively identified as *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1).

The isolates' inherent degradative properties were exploited in an attempt to increase cell yield for plasmid preparations. However, rich media were eventually used to prepare large volumes of cell material quickly. *Acinetobacter* (8W-1) was known to be limited to degradation of biphenyls (Fedorak and Westlake, 1983) and this degradation appeared to be inducible by growth on biphenyls. *Aeromonas* (38W-HC10-1) was capable of limited metabolism of some aromatic compounds; for example, phenanthrene was oxidized but not mineralized, and could not serve as a sole carbon and energy source. *Flavobacterium* (8W-2) had the most extensive aromatic-degrading capability, being able to mineralize phenanthrene, as well as smaller aromatics, and being able to partially oxidize compounds such as anthracene and dibenzothiophene. These conclusions were inferred from both ^{14}C studies and GC results.

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Chapter 3

Plasmid Preparation Techniques

3.1 Introduction

The first plasmid preparation techniques reported in the literature were designed for use specifically with *E.coli* carrying small (less than 10 Mdal) plasmids. These techniques were not suitable for strains carrying degradative plasmids because of their larger size, and first attempts to visualize degradative plasmids like TOL in pseudomonads failed (Palchaudhuri and Chakrabarty, 1976).

As interest in degradative plasmids grew, so did the number of papers outlining plasmid preparation modifications, which were usually designed for ATCC and NCIB strains of *Pseudomonas*. Modifications usually included lysis of cells at high pH to denature sheared chromosomal DNA, leaving supercoiled plasmid DNA intact. Phenol extractions were sometimes included to disrupt membrane-DNA associations. These modifications were found to be necessary since the larger molecular weight plasmids tend to become associated with both chromosomal DNA and cell membranes, making purification difficult (Palchaudhuri and Chakrabarty, 1976).

In recent years, techniques have been published which were designed to isolate large plasmids from genera such as *Rhizobium* spp. (Casse *et al*., 1979), *Vibrio* spp. (Hada and Sizemore, 1981), *Agrobacterium* spp. (Kado and Liu, 1981)

Alcaligenes spp. (Kiyohara *et al.*, 1981), and *Beijerinckia* spp. (Kiyohara *et al.*, 1983). These have proven very useful to researchers working with non-laboratory strains.

In the course of this project, an attempt was made to find a reproducible procedure for visualization of PAH degradative plasmids from a variety of environmental isolates. Ease of preparation and adaptation to quick screening techniques were desireable, but were secondary in importance to reliability. Unmodified plasmid preparation techniques were usually successful with genera such as *Aeromonas*, *Acinetobacter*, and the standard *Pseudomonas* and *E. coli* cultures, but unsuccessful with *Flavobacterium* (8W-2). Because the focus of the thesis fell on the latter isolate, the literature was searched to find a procedure designed for *Flavobacterium*. The search was unsuccessful because little or no genetic work has been published on this genus. Therefore a number of different plasmid preparation methods and modifications were used to attempt plasmid isolation; these techniques are presented here. Modifications to the technique of Casse *et al.* (1979) allowed reproducible isolation of the *Flavobacterium* (8W-2) plasmids.

3.2 Methods and Materials

A variety of Tris - EDTA (TE) and Tris - EDTA - NaCl (TES) buffers are specified in the procedures reported here. To differentiate the buffers, the concentrations of their components are prefixed to each abbreviation (e.g. 50/10/100 TES refers to 50mM Tris - 10mM Na_2EDTA - 100mM NaCl).

The final pH of alkaline lysis mixtures originally was measured with a glass electrode and Fisher pH Meter calibrated to pH 12.5 with saturated $Ca(OH)_2$, but finally was routinely judged using plastic-backed pH indicator strips (Merck), comparing the color of the strip with the standard chart provided with the package.

3.2.1 Large-scale Preparation

This procedure, as used in J.Brunton's laboratory (Dept. Medical Microbiology, University of Alberta, 1981), was used to prepare large volumes of standard *E. coli* plasmid DNA for reference purposes in this project.

The pellet from a 200mL overnight culture was washed in 50/5 TE (50mM Tris - 5mM Na_2EDTA, pH 8.0) and resuspended in 12.5mL TE - 25% sucrose (pH 8.0). Then 1.75mL lysozyme (5mg/mL freshly prepared in 0.25M Tris, pH 8.0) was added and the mixture was incubated in ice for 5 min. A 6.25mL volume of 0.25M Na_2EDTA (pH 8.0) was mixed in, then incubated at 0°C for 10 min. Lysis was brought about by the addition of 12mL of lytic mixture (50mM Tris - 50mM Na_2EDTA - 2% v/v Triton X-100 - 50μg/mL heat-treated RN'ase, pH 8.0) and incubation in ice for 30 min. The lysate was centrifuged at 19,000 rpm (43,500 x g) at 4°C for 40 min to pellet debris. The supernatant was retained, and DNA precipitated by adding 0.11 volumes of 3M sodium acetate plus 2 volumes of 95% ethanol, and chilling at -20°C overnight. The DNA was recovered by centrifuging at 10,000 rpm (12,100 x g) at -20°C for 20 min. The DNA pellet was resuspended in 5mL 50/5/150 TES, extracted with chloroform, and centrifuged at 15,000 rpm (27,000 x g) at 4°C for 30 min, retaining the upper aqueous phase. Cesium chloride was added to the aqueous phase at 1g/mL with 0.2mL of ethidium bromide (EtBr; 10mg/mL in 50/5/150 TES), after which the mixture was protected from light. The refractive index of the solution was adjusted to 1.3878. The solution was centrifuged in "Ultraclear" (Beckman) preparative centrifuge tubes in a Ti50 rotor at 40,000 rpm (105,500 x g) for 40 to 48 hr. DNA bands were visualized with ultraviolet light. The lower, plasmid band was recovered either by collecting drops or by withdrawing material through the side of the tube with a syringe. Ethidium bromide was removed by multiple extractions with CsCl-saturated iso-propanol. The remaining solution was diluted to 10mL with 50/5 TE and precipitated with sodium acetate/ethanol, chilled overnight at -20°C and collected by centrifugation, as before. The pellet was drained, dried, and resuspended in 200µL 50/5/150 TES for visualization.

3.2.2 Zuniga et al. (1981) Procedure

This rapid screening procedure was used to test *P. putida* (ATCC 33015) for plasmid content during curing experiments in this project.

With a sterile toothpick, a portion of a colony was resuspended in 50µL of 25% sucrose - 50mM Tris (pH 8.0) plus 2µL RN'ase (5mg/mL in 0.25M Tris, pH 8.0) plus 4µL 0.25M Na_2EDTA plus 6µL lysozyme (5mg/mL in 0.25M Na_2EDTA, autodigested 30 min at 37°C) in a 1.5mL microfuge tube. This mixture was incubated at room temperature for 20 min. Then 4µL of 20% SDS in 10mM Tris (pH 8.0) was added and incubated for 2 min at room temperature. The tubes were placed on ice for 1 hr, after which they were equilibrated to room temperature and mixed. Sufficient loading buffer (Appendix) was added to increase sample density for agarose gel electrophoresis.

3.2.3 Meyers et al. (1976) Variation #1

The technique of Meyers *et al*. (1976) has been modified in many laboratories. The first variation reported here is a modification of a technique used in the lab of S.Spiegelman (Dept. Microbiology, University of British Columbia, 1980). The procedure was used in this project for initial plasmid screening experiments.

Cells from 200mL of broth culture were harvested and resuspended in 2mL of 25% sucrose - 10mM Tris - 1mM Na_2EDTA (pH 8.0). All subsequent steps were carried out in 25mL glass centrifuge tubes with screw cap closures. Freshly prepared lysozyme (5mg/mL in 0.25M Tris, pH 8.0) was added at 0.2mL/mL resuspended culture; the mixture was incubated on ice for 10 min. Stock SDS solution (10%w/v) was added to a final concentration of 1% and lysis was initiated by incubation in a 60°C waterbath for 3 to 5 min with hand mixing. Fifty microlitres of heat-treated RN'ase (1mg/mL in 50mM sodium acetate, pH 5.0) was added to the lysate, and incubated at 37°C for 1 hr. Three-tenths millilitre of Pronase® (10mg/mL in 10mM Tris, pH 7.4; autodigested for 30 min at 37°C) was added and incubated at 37°C for 30 min, after which time the mixture was gently mixed with a vortex to shear chromosomal DNA. Alkaline denaturation of chromosome fragments was effected by adding fresh 3N NaOH dropwise to a final pH of 12.1 to 12.3. The viscous solution was mixed well by hand for 10 min. Single stranded chromosomal DNA fragments generated by denaturation were precipitated by neutralizing the solution with dropwise addition of 2M Tris (pH 7.2) to pH 8.5 to 9.0 with hand mixing for 5 min, then salted out by adding 0.4mL of 30% NaCl (final concentration 3% w/v) with gentle mixing. Finally, 1 volume of "equilibrated phenol" (melted crystalline phenol mixed and equilibrated at 60°C in 2:3 ratio with 3% aqueous NaCl) was added and mixed thoroughly for 10 min to partition the single stranded DNA into the phenol phase. Centrifugation at 7,000 rpm (6,000 x g) for 5 min separated the phases so that the upper aqueous phase could be removed with a pipette. The DNA in the aqueous phase was precipitated by the addition of 2 volumes of cold 95% ethanol and overnight incubation at -20°C. The precipitated DNA, enriched for covalently closed plasmids, was collected by centrifugation and dried at room temperature to remove all ethanol.

3.2.4 Meyers et al. (1976) Variation #2

A second variation of this technique was used in J.Brunton's laboratory (Dept. Medical Microbiology, University of Alberta, 1980), and used in this project for initial plasmid screening experiments.

Growth from 3 agar plates was washed, collected, and resuspended in 1.5mL of 25% sucrose - 20mM Tris - 2.5mM Na_2EDTA (pH 8.0). Two-tenths millilitre lysozyme (5mg/mL in 0.25M Tris, pH 8.0) was added, and incubated at room temperature for 5 min. Then 0.4mL of 0.25M Na_2EDTA was added with hand mixing and incubated at room temperature for 20 min. After the addition of 0.6mL of 5M NaCl, the solution was incubated at 4°C overnight. The lysate was cleared by centrifugation at 17,000 rpm (34,800 x g) at 4°C for 35 min to pellet chromosomal DNA. The supernatant, containing plasmid DNA, was doubled in volume by the addition of 2.95mL distilled water, then digested with the addition of heat-treated RN'ase (final concentration of 20µg/mL), and incubating for 30 min at 22°C. Two equilibrated liquid phenol extractions were followed by a chloroform extraction, then the DNA in the aqueous phase was precipitated by the addition of 2 volumes of 98% ethanol with overnight incubation at -20°C. DNA was collected by centrifugation at 15,000 rpm (27,000 x g) for 15 min at -11°C.

3.2.5 Variation on Hansen and Olsen (1978) Technique

The technique of Hansen and Olsen (1978) has been used as a basis for modified plasmid isolation techniques in many laboratories. It was modified in this project for use with the *Flavobacterium* (8W-2) sp. in the following manner. Using glass screw cap centrifuge tubes, cells from 50mL of culture were harvested, washed in 10mL of 10mM sodium phosphate buffer (pH 7.0), and re-pelleted. This pellet was resuspended in 1.35mL of 25% sucrose - 50mM Tris (pH 8.0) at room temperature. One-tenth millilitre of lysozyme (10mg/mL in 0.25M Tris, pH 8.0) was added and incubated in ice for 5 min. Mixing from this point was done by slow inversion of the tubes to minimize shear force on the lysate. A 0.5mL volume of 0.25M Na_2EDTA (pH 8.0) was added, mixed by inversion, and incubated in ice for 5 min. Lysis was effected by adding 0.5mL of 20% SDS in 50/20 TE (50mM Tris - 20mM Na_2EDTA, pH 8.0). A heat pulse cycle of 15 sec heat at 50°C and 15 sec inversion mixing was repeated 8 times. The pH of the lysate was increased to between 12.1 to 12.3 by addition of 0.5mL freshly prepared 3N NaOH. Neutralization with 1mL of 2M Tris (pH 7.2) in two equal parts was followed by inversion mixing. This dropped the pH to 8.5 to 9.0. Then

0.65mL of stock SDS (20% SDS in 50/20 TE) was added, followed by 1.25mL of 5M NaCl and 20 inversions. The lysate was incubated in an ice water bath at 4°C overnight. After this salting out period, the lysate was centrifuged at 12,000 rpm (17,300 x g) for 30 min at 4°C. The supernatant was poured into chilled graduated conical plastic centrifuge tubes to remove any flocculated material and to measure the volume. The supernatant was decanted to another plastic centrifuge tube, 0.313 volumes of polyethylene glycol 6000 (42% w/v in 10mM sodium phosphate buffer, pH 7.0; final concentration 10%) was added, and the mixture was stirred gently. After overnight incubation at 4°C the mixture was centrifuged at 2,500 rpm (755 x g) for 5 min at 4°C. The pellet was saved, resuspended in 0.15mL cold 50/5/50 TES (50mM Tris - 5mM Na_2EDTA - 50mM NaCl, pH 8.0) and resolved on agarose gels for visualization.

3.2.6 Variation on Birnboim and Doly (1979) Technique

The following modification of the method of Birnboim and Doly (1979) was intended to provide a quick plasmid screen for this project. All steps were carried out in 1.5mL microfuge (Eppendorf) tubes. One-half millilitre of culture was pelleted in a microfuge at top speed (approximately 11,000 x g) for 1 min. The pellet was resuspended in 100μL of Solution I (2mg/mL lysozyme - 50mM glucose - 10mM Na_2EDTA - 25mM Tris, pH 8.0) and incubated in ice for 30 min. Two hundred microlitres of freshly prepared Solution II (0.2N

NaOH in 1% SDS) were added and gently mixed with a vortex to achieve lysis. The lysate was incubated on ice for 5 min and then 150μL of 3M sodium acetate (pH 4.8) was added, with further incubation on ice for 1 hr. The tubes were centrifuged for 5 min at top speed in a microfuge. Then 0.4mL of the supernatant was transferred to another microfuge tube and precipitated by the addition of 1.0mL of ice cold 95% ethanol. Collection of the DNA was completed by incubating at -20°C for 30 min then centrifuging at top speed for 2 min in a microfuge. Pellets were drained, dried, and reprecipitated in sodium acetate/ethanol. The resultant pellet was air dried and dissolved in approximately 40μL water for visualization on an agarose gel.

3.2.7 Kado and Liu (1981) Modification

The following modification was used occasionally as a rapid screen for determining the plasmid content of phenotypic variants generated in this project. In this procedure, either 1.5mL of an overnight culture or several colonies from an agar plate were pelleted in a microfuge tube and the pellet was washed with 50/20 TE (50mM Tris - 20mM Na_2EDTA, pH 8.0) and resuspended in 100μL lysing solution (3% SDS in 50mM Tris, pH 12.6). The cells were lysed at 55°C for 20 min, followed by extraction with an equal volume of melted crystalline phenol:chloroform (1:1). It is important that the phenol be very pure; reagent grade is inadequate (C.I.Kado, personal communication, 1983). The phases were separated by 5 min centrifugation in a microfuge. The upper aqueous phase was removed with an Eppendorf pipette. This sample could be used immediately, or the phenol could be removed by extraction with ethyl ether, and the DNA precipitated with sodium acetate/ethanol for restriction analysis.

Modifications of this procedure included increasing the lysis solution volume to 200μL, and increasing the SDS concentration to 4% to achieve lysis. As well, the short precipitation time for plasmid recovery from ethanol was lengthened from the recommended 5 min in dry-ice/ethanol to an overnight incubation at -20°C.

3.2.8 Clewell and Helinski (1970) Modification

This procedure uses Triton X-100 instead of SDS to achieve cell lysis. It was used for studies reported in Chapter 5 where the use of a non-ionic detergent was indicated, and also as an alternative to SDS lysis prior to CsCl buoyant density centrifugation.

Eleven millilitres of an overnight culture (approximately a 150mg pellet) was washed once in 50/20 TE buffer (50mM Tris - 20mM Na_2EDTA, pH 8) and resuspended in 12mL of 25% sucrose - 50mM Tris (pH 8). One-tenth millilitre lysozyme (5mg/mL in 0.25M Tris, pH 8) was added and incubated in ice for 5 min. To achieve lysis, 1mL of 0.25M Na_2EDTA was added and the solution was incubated in ice for 5 min. An equal volume (approximately 12mL) of 50/20 TE - 1%

Triton X-100 was mixed with the cell suspension. The mixture was incubated at room temperature until lysis was apparent, about 8 min. The lysate was centrifuged at 15,000 rpm (27,000 x g) for 20 min, and the supernatant decanted for ethanol precipitation. Alternatively, the supernatant could be phenol extracted prior to precipitation.

3.2.9 Portnoy and White (1981) Modification

The Portnoy and White procedure was cited by Crosa and Falkow (1981) as a personal communication. It is a small culture volume procedure suitable for screening large numbers of cultures. The basic procedure is presented first, followed by various modifications specifically used for *Flavobacterium* (8W-2).

A 1.5mL volume of overnight culture was harvested at top speed (approximately 11,000 x g) in a bench-top microfuge, washed once in 50/10 TE (50mM Tris - 10 mM Na_2EDTA, pH 8.0) and resuspended in 40μL 50/10 TE. Lysis buffer was freshly prepared (4% SDS in 50/10 TE, pH 12.4 determined approximately by plastic pH indicator strips; Merck), and 0.6mL was added to the cell suspension. The suspension was mixed by tapping the tube or by gentle vortical mixing, then incubated at 37°C for 20 min to achieve lysis. The suspension was neutralized by adding 30μL of 2.0M Tris (pH 7.0), mixing by inverting the tubes until viscosity was visibly reduced. Chromosomal DNA was salted out by the addition of 0.24mL of 5M NaCl (final concentration of approximately 1M NaCl) with complete mixing. The solution was placed on ice for a minimum of 1 hr, then centrifuged at top speed in a microfuge for 10 min to sediment debris. The supernatant was precipitated by the addition of 0.55mL of iso-propanol and cooling to -20°C for 30 min. The DNA was collected by centrifugation in a microfuge for 3 min. After air-drying, the pellet was resuspended in 30µL 50/5/50 TES (50mM Tris - 5mM Na_2EDTA - 50mM NaCl, pH 8.0) for electrophoresis. Variations on the Portnoy and White method included: age and volume (from 1.5 to 6mL) of culture; lysis time (from 10 to 40 min) and temperature (from 37°C to 55°C); use of freeze-thaw cycles to aid lysis; altered concentration of SDS in lysis buffer (from 3% to 8%) or use of lysozyme; thoroughness of mixing (i.e. shear forces employed); volume of neutralizing solution (30µL to 300µL); and final pH achieved after neutralization; volume of 5M NaCl used for salting out chromosomal DNA (0.24 to 0.28mL); duration of salting out period (1 hr to overnight); temperature of salting out (0°C to 4°C); precipitation in iso-propanol versus sodium acetate/ethanol for 30 min in dry-ice acetone or overnight at -20°C; horizontal versus vertical agarose gels for visualization.

3.2.10 Wheatcroft and Williams (1981) Modification

These researchers addressed the problem of isolation of plasmids which had proven refractory to other isolation procedures. Using their rapid method of extraction, they were able to isolate plasmids previously undetected. The procedure was used in this project to isolate plasmid DNA sufficiently pure for restriction endonuclease digestion.

Fifty microlitre volumes of culture were harvested and resuspended in 2mL of 50/50 TE (50mM Tris - 50mM Na_2EDTA, pH 8.0). A 1.6mL aliquot of this suspension was removed to a 13mm x 100mm silanized glass test tube and gently mixed with a vortex while 0.4mL of 1M NaOH saturated with SDS was slowly added. The pH of the resultant solution appeared to be 12.5, using plastic pH indicator strips (Merck). The lysates, which were very viscous, were then mixed on a vortex at high speed for 5 min to shear chromosomal DNA. If foaming from agitated SDS was excessive, a small injection of Antifoam A spray (Dow Corning) was used in each tube. Sucrose gradients were prepared by two freeze-thaw cycles of 11.5mL 20% sucrose in distilled water using Ultraclear (Beckman) preparative centrifuge tubes. Cell lysates were layered on thawed gradients and centrifuged in a Beckman preparative ultracentrifuge with a swinging bucket rotor (SW40.1) at 28,000 rpm (100,000 x g) for 1 to 1.5 hr at 20°C with no braking. Fractions of approximately 0.75mL were collected by puncturing the bottom of the tube. Aliquots of these fractions were assayed for plasmid content by agarose gel electrophoresis, and those fractions enriched for plasmid DNA were suitable for restriction digests without further treatment.

3.2.11 Casse et al. (1979) Variations

The modified procedure as it was originally used to isolate plasmids from *Flavobacterium* (8W-2) is presented first. Extensive changes to the procedure resulted in the final "modified Casse procedure".

3.2.11.1 First Version

Two hundred millilitres of 24 hr cultures were harvested, resuspended in 20mL of 1M NaCl and incubated with shaking for 15 min at room temperature. The cells were washed twice in 50/20 TE (50mM Tris - 20mM Na_2EDTA, pH 8.0) in glass centrifuge tubes, and the pellet weight was adjusted to approximately 200mg. The cells were resuspended in 1mL 50/20 TE and 9.5mL lysis solution (4% SDS in 50/20 TE, pH 12.4) was added and gently mixed by inversion. The suspension was incubated at 37°C for 20 min to achieve lysis, at which point the solution was clear and very viscous. The lysate was neutralized with 1mL of 2M Tris (pH 7.0) to reduce the pH to 8.0-8.5, mixing well by slow inversions until the solution became less viscous. A small, undetermined weight of Pronase® (from *Streptomyces griseus*; Calbiochem) was added and the mixture was incubated at 37°C for 20 min. Alternatively, Proteinase-K, Type XI (Sigma) was used under the same conditions. Then 0.3g of NaCl (final concentration approximately 3% w/v) was added, mixed, and incubated at 37°C for 1.5 hr to salt out denatured chromosomal DNA. Ten millilitres of liquefied phenol (90% phenol, Fisher) equilibrated with 1M NaCl (aqueous), was added and mixed by inversion. The phases were separated by centrifugation at 6,500 rpm (5,000 x g) for 10 min at 4°C. The upper aqueous phase was removed with a Pasteur pipette and precipitated by the addition of 0.11 volume of 3M sodium acetate and 2 volumes of 95% ethanol with subsequent cooling to -20°C overnight. The precipitated DNA was collected by centrifugation at 10,000 rpm (12,100 x g) for 10 min at 4°C, air dried, and dissolved in 2mL 50/20 TE for electrophoresis.

3.2.11.2 Modifications to the first version

The age of the *Flavobacterium* (8W-2) culture was varied from 17 to 72 hr in STCS broth, (grown at 26°C in flasks) and the cell pellet weight was varied from 50mg to 250mg wet weight. Use of stored cells (eg. frozen pastes stored at -70°C or liquid cultures stored at 4°C) was attempted. Pre-incubation of the pellet with NaCl before lysis was discontinued. Resuspension of the pellet in 50/20 TE was accomplished with 0.5mL for the smaller pellets, rather than the 1mL in the original version. Lysis times and temperatures were varied from 37°C to 55°C for 5 to 90 min. Neutralization was enhanced by a more vigorous mixing of the viscous lysates by briefly mixing with a vortex at lowest speed. Pronase® and proteinase treatments were abandoned. The amount of NaCl used to salt out chromosomal DNA was varied: solid NaCl (from 300 to 500mg) and 5M NaCl solution (f.c. 1M) were tried. Salting out times and temperatures were varied from 20 min to 2 hr. Different qualities of phenol were used for extractions: liquefied (90%, Folin-Ciocalteau Reagent; Fisher); pure crystalline (Mallinckrodt or BDH); or ultrapure (BRL) melted at 55°C were used. Storage of the phenol was varied: liquid phenol was stored at room temperature; pure crystalline phenol was stored either at room temperature or at -20°C; ultrapure phenol was stored at -20°C under N_2. The amount of mixing of phenol and 50/20/100 TES prior to extraction was varied, as was the shear force during mixing of the plasmid preparation with the phenol. The use of Teflon® tubes for leak-proof mixing during this stage was adopted. In some cases, $CHCl_3$ was used in the phenol extraction step (1:1 with equilibrated phenol) to enhance phase separation. In other cases, the extracted phenol phase (± $CHCl_3$) was re-extracted with fresh TE as a "back-extraction" to improve recovery of plasmids from interfacial material. Precipitation of the plasmid preparation in sodium acetate/ethanol at -20°C was carried out for 1 to 72 hr to determine the optimum time for plasmid recovery. Plasmids were dissolved in distilled water or 10/4 TE or 50/20 TE. The volumes varied according to the amount of starting material and apparent yield of DNA after precipitation.

Different combinations of these modifications led to optimized plasmid recovery from *Flavobacterium* (8W-2), as summarized in the final version of the Casse *et al.* (1979) procedure.

3.2.11.3 Final Modified Casse Procedure

Sterile Teflon® screw cap centrifuge tubes (Sybron) were used throughout the procedure. Ten millilitres of overnight culture (18 to 24 hr, grown in STCS shake flasks) were harvested, washed once in 50/20 TE and pellet weight adjusted to no more than 100 to 150mg. The pellet was resuspended in 0.5mL 50/20 TE, and 9.5mL of lysing solution [4% SDS in 50/20 TE; pH approximately 12.4 measured by pH strips (Merck)] was added with mixing. The cells were lysed at 55°C for 10 min, then neutralized by the addition of 1mL 2M Tris (pH 7.0) (final pH approximately 8, measured with pH indicator sticks) with very thorough mixing. The chromosomal DNA was salted out by adding 0.33g NaCl with thorough mixing, then letting the solution sit at room temperature for 30 min. Melted crystalline phenol (BDH) (equilibrated in equal volumes with 50/20/1000 TES at 55°C) was added and mixed with the aqueous phase by rapid inversions. The phases were separated by centrifugation at 5,000 rpm (3,020 x g) for 10 min at 4°C. Eight millilitres of the upper phase was removed with a wide-mouth plastic Eppendorf pipette, avoiding interfacial material. The 8mL volume was placed in another sterile Teflon® tube and the DNA was precipitated by the addition of 0.11 volumes of 3M sodium acetate and 2 volumes of 95% ethanol, chilling the liquid to -20°C for 18 to 24 hr. The precipitate was collected by centrifugation at 10,000 rpm (12,100 x g) at -14°C for 10 min. The pellet was rinsed with 95% ethanol, then air-dried. Two hundred microlitres of 10/4 TE was used to dissolve the pellet, of which 20 to 40μL was used for electrophoresis.

3.2.12 Plasmid Visualization

The plasmid preparation was dissolved, if necessary, in a small volume of appropriate dilute TE. The sample density was increased by adding a loading buffer, which could be 16% Ficoll in dilute TE plus a trace of Bromphenol Blue, or 33% glycerol-7% SDS in dilute TE with Orange G as a marker. Loading buffers were usually added to a final concentration of about 20% v/v.

Gels were prepared by dissolving 0.7% w/v agarose (Seakem LE; Marine Colloids, Rockland, ME) in running buffer, usually 0.5M Tris - 10mM Na_2EDTA - 0.5M boric acid (TEB), or 0.5M Tris - 10mM Na_2EDTA - 0.1M sodium acetate - 0.9M NaCl (TEAS), by heating to boiling in a microwave oven. The agarose solution was cooled to about 60°C and poured into vertical gel boxes or into a horizontal gel apparatus (Bethesda Research Laboratories). A double strength gel was used as a support for the vertical gels. Usual electrophoresis conditions for 20cm x 12 cm vertical gels were 100v, constant voltage, for 3 hr. This voltage usually produced a current of 20 to 25 mamp in TEB. After electrophoresis, gels were removed from the apparatus and stained in a dilute EtBr solution of approximately 10µg/mL, usually for 30 min to 1 hr. The stained gels were observed on an ultraviolet transilluminator box (UltraViolet Products) and photographed through a red celluloid filter with a Polaroid Land camera using Polaroid Type 52 or Type 665 film. Usual exposure time was 1 to 2 min.

3.3 Results and Discussion

The large scale CsCl centrifugation method developed in J.Brunton's laboratory (Dept. Medical Microbiology, University of Alberta) was used in this project to successfully isolate plasmids from cultures carrying single plasmids [*E. coli* standard cultures with R plasmids; *P. putida* (ATCC 33015) carrying TOL] and *Acinetobacter* (8W-1) carrying multiple small plasmids. However, when *Flavobacterium* (8W-2) was centrifuged by this method, a single band was observed in the CsCl gradient, regardless of procedure modifications. Aliquots of this band electrophoresed in 0.7% agarose gels showed only material co-migrating with chromosomal DNA. It is possible that the *Flavobacterium* (8W-2) plasmids were not adequately separated from chromosomal material by this method and therefore migrated with the chromosomal DNA in the gradient. Alternatively, the plasmids may nave been linearized by the procedure, causing them to co-migrate with linear chromosomal DNA.

The rapid screening technique of Zuniga *et al*. (1981) was used successfully in this laboratory for isolating plasmids from *P.putida* (ATCC 33015) when screening mutants for plasmid content. It was not consistently successful in isolating plasmids from *Flavobacterium* (8W-2). The Meyers *et al*. (1976) variations, and Birnboim and Doly (1979), Kado and Liu (1981) and Hansen and Olsen (1978) modifications also proved unsatisfactory for use with the isolates studied in this project because of inconsistent plasmid isolation, and were not routinely used. The Triton X-100 "cleared lysate" method of Clewell and Helinski (1970) was not effective in isolating plasmids from *Flavobacterium* (8W-2), probably because the neutral lysis conditions did not permit adequate separation of chromosomal and plasmid DNA. (However, the non-ionic surfactant lysis technique was useful for preparing cells for CsCl centrifugation.)

Extensive modification of the Portnoy and White (1981) technique (as cited by Crosa and Falkow, 1981) revealed several important factors; the most important of these were the use of young cultures (less than 48 hr old) and the use of extended salting out times to remove chromosomal DNA. Thorough mixing was necessary, especially since the microfuge tubes used in this procedure afforded poor mixing conditions when they contained large volumes of liquid. The mass of cells used in the preparation had to be optimized:

too little cell material meant a very faint band on agarose gels, while too many cells provided an excess of chromosomal material which seemed to interfere with plasmid isolation, as noted by Mickel *et al*. (1977). Vertical electrophoresis provided a superior gel when compared with horizontal electrophoresis. Despite application of these various modifications, plasmid isolation by this technique was inconsistent even with *E.coli* standards, and the procedure was not studied further.

The sucrose centrifugation method of Wheatcroft and Williams (1981) was rapid (2 hr) and plasmid bands could be used immediately for restriction or transformation attempts (although, of course, multiple plasmid species migrated as a single band). The method was used successfully for plasmid isolation from *Aeromonas* (38W-HC10-1) because the single, large plasmid was easily separated from chromosomal DNA in a 1 hr centrifugation. Success with *Flavobacterium* (8W-2) using this method was variable, and sometimes the plasmid fraction appeared to be contaminated with chromosomal DNA. It is probable that the vortexing step, used to shear chromosomal material in this procedure, linearized *Flavobacterium* (8W-2) plasmid DNA. This was inferred when aliquots from sucrose gradient fractions were run in parallel 0.7% and 1.2% agarose gels. In the standard 0.7% gel, a large amount of contaminating chromosomal DNA was present. However, in the 1.2% agarose gel, all of this apparently chromosomal material migrated in a discrete band above authentic chromosomal DNA present in control lanes (data not presented). This suggested that the material was actually linearized *Flavobacterium* (8W-2) plasmid DNA which co-migrated with chromosomal DNA in the 0.7% agarose gels normally used to observe sucrose gradient fractions. Therefore, it is likely that many sucrose gradients were discarded as being unsuccessful when the DNA could have been retained for restriction analyses.

3.3.1 Effects of modifications to the Casse et al. (1979) procedure

The modified Casse procedure, originally designed to isolate high molecular weight plasmids of *Agrobacterium*, *Pseudomonas*, and *Rhizobium* (Casse *et al.*, 1979), proved to be the most reliable method for isolating plasmids from the environmental isolates used in this thesis, especially *Flavobacterium* (8W-2). All PAH degrading isolates tested, except isolate 20S-8, bore at least one plasmid. Plate 3.1 shows the plasmid profiles of wild type *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) produced by the modified Casse technique. Although the unmodified procedure was adequate for *P. putida* (ATCC 33015), *Aeromonas* (38W-HC10-1) and *Acinetobacter* (8W-1), extensive modification of individual steps was necessary to optimize conditions for *Flavobacterium* (8W-2) plasmid isolation. A recent publication by Hogrefe and Friedrich (1984) documenting isolation procedures for

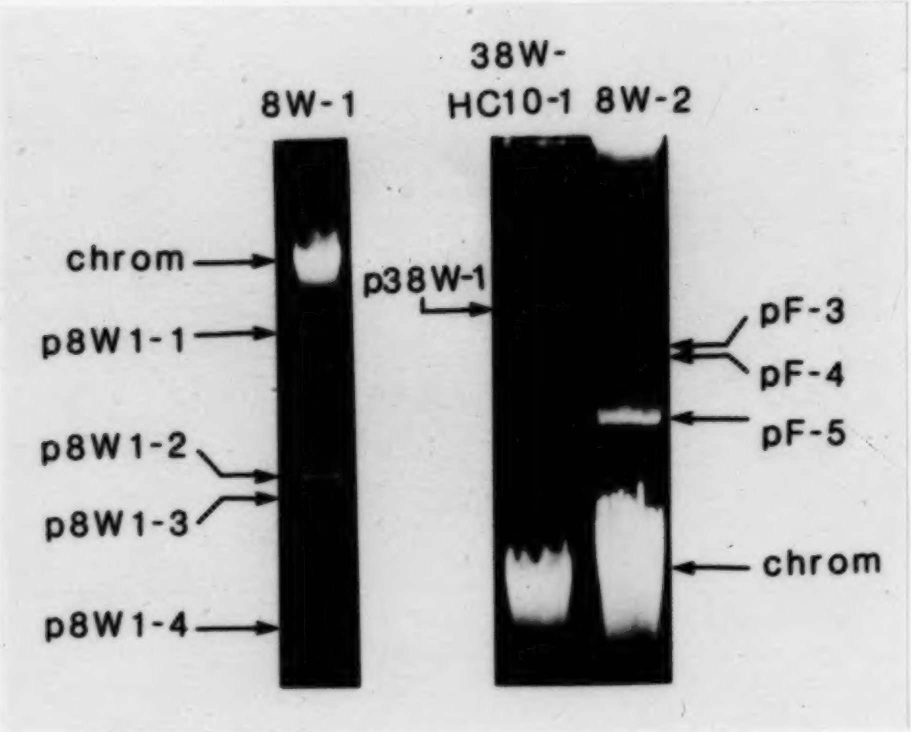
38W-
HC10-1
8W-1
8W-2
chrom
p8W1-1
p8W1-2
p8W1-3
p8W1-4
p38W-1
pF-3
pF-4
pF-5
chrom

Plate 3.1 Photograph of plasmids of *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) wild types, prepared by the modified Casse method. Apparent molecular weights determined by comparison with known plasmids: p8W1-1 (17Mdal); p8W1-2 (9Mdal); p8W1-3 (7Mdal); p8W1-4 (5Mdal); p38W-1 (122Mdal); pF-3 (74Mdal); pF-4 (55Mdal); pF-5 (30Mdal).

"megaplasmids" from various lithotrophs substantiates a number of the observations made during modifications to the Casse technique.

Both the pre-lysis salt-incubation step and the post-lysis Pronase® (or proteinase-K) digestion steps were discontinued, since their inclusion did not enhance plasmid recovery (Chapter 5). The age of the *Flavobacterium* (8W-2) culture used for plasmid preparation was an important factor in successful plasmid isolation, as had been observed with the Portnoy and White method. This was demonstrated with plasmid preparations performed on cells from Fermenters "A" and "B" (Chapter 2). The fermenters had the same inoculum and were grown under identical conditions, except that Fermenter "B" was harvested after 24 hr growth, while Fermenter "A" was harvested after 42 hr. The younger cells (Fermenter "B") consistently gave good plasmid bands, while successful plasmid preparations from the older cells (Fermenter "A") were never achieved. Repeated experiments showed that *Flavobacterium* (8W-2) cells grown for longer than 26 hr (in STCS at 26°C) were unsuitable for plasmid preparations by the modified Casse procedure. Those *Flavobacterium* (8W-2) cultures incubated for 17 to 24 hr with A_{600} values from 3.8 to 5.5 were suitable. Similarly, *Aeromonas* (38W-HC10-1) cultures 24 hr old were superior to 48 or 72 hr cultures in Casse plasmid preparation. One can hypothesize that the 24 hr culture was undergoing exponential growth, during which time the copy number of large plasmids would be maximal, being integrated with chromosomal replication. This increased copy number would enhance the isolation of large plasmids. There might also be membrane-associated phenomena in dividing cells such as membrane attachment of large plasmids or the degree of supercoiling of chromosome/plasmid DNA during continuous replication that might be reflected in ease of plasmid isolation. Another possibility is the production of DN'ases in older (late log or stationary) cultures which might affect plasmid preparation. Although the reasons were not explored, it was observed that young cultures consistently provided better material for plasmid preparations than old cultures.

The initial mass of cell material also was found to be important, as observed by Mickel *et al*. (1977) and Hogrefe and Friedrich (1984). Pellets of 50 to 100mg (wet weight) were superior to larger pellets for all strains tested. This weight usually corresponded to overnight culture volumes of approximately 2mL for *P. putida* (ATCC 33015) or *Aeromonas* (38W-HC10-1), 5 to 8mL for *Flavobacterium* (8W-2), and 20mL for *Acinetobacter* (8W-1). It is assumed that the large plasmids are present in low copy number. Therefore, a large starting cell mass would tend to overwhelm the plasmid preparation with excessive chromosomal material. In fact, it was observed during procedure manipulations that very viscous lysates invariably gave very poor plasmid profiles. Part of the problem was, no doubt, physical, caused by insufficient mixing of the neutralizing solution, NaCl, and phenol (Hogrefe and Friedrich, 1984). Another problem caused by excessive chromosomal material was streaking in the lanes of agarose gels, with occasional obscuring of the plasmid band by chromosomal DNA.

The effect of cell storage on plasmid isolation was investigated using *Flavobacterium* (8W-2). Cell pellets frozen in a "paste" at -20 or -70°C were suitable for plasmid preparations, but cells stored as liquid cultures at 4°C were invariably poor sources of plasmids. There was no apparent difference in the quality of plasmid preparations using cells stored at -20°C or -70°C.

Lysis times and temperatures were varied to determine their effect on plasmid isolation from *Flavobacterium* (8W-2). Large plasmids associate with the cell membrane and host chromosome (Palchaudhuri and Chakrabarty, 1976), and disruption of the association is necessary for successful plasmid isolation. Heating with SDS (in the lysis mixture) should enhance this dissociation; therefore, the original lysis temperature of 37°C was increased to 55°C, which improved lysis of *Flavobacterium* (8W-2) cells, and yielded marginally better plasmid preparations. This phenomenon was also observed by Hogrefe and Friedrich (1984) with various lithotrophs.

The lysis pH is crucial to plasmid recovery, regardless of host cell genus. At pH 12.4 to 12.5, sheared chromosomal DNA will denature into single stands, whereas covalently closed, supercoiled plasmid DNA will not denature (Birnboim and Doly, 1979). Lysis at too low a pH value will not denature sufficient chromosomal DNA to enrich the preparation for plasmids, while too high a pH will denature the plasmids. Plastic pH indicator strips (Merck) seemed to be adequate for reproducibly adjusting the pH of the lysis mixture. The lysis mix was freshly prepared for each plasmid preparation, since storage lowered the pH (probably by absorption of CO_2).

Lysis time was limited to 10 min at the higher temperatures, which was usually sufficient for a visible clearing of the cell suspension with the strains tested. In rare instances when this temperature was insufficient for effecting lysis of *P. putida* (ATCC 33015) or *Aeromonas* (38W-HC10-1), heat pulses were used to effect lysis. In the case of the *E.coli* standards, lysozyme was employed along with heat pulses at 65°C.

Proper mixing of the lysate with neutralizing solution was found to be necessary. Improper mixing resulted in localized deviations from the desired pH of 8.5 to 9, especially with viscous preparations. Therefore, gentle mixing of the lysate with neutralizing solution (by inversion or with a vortex at low speed) was performed until the solution appeared homogeneous and had become less viscous. The addition of NaCl to the neutralized lysate was intended to salt out the sheared, alkali-denatured chromosomal DNA. Sodium chloride (solid or as a 5M solution) was added to to the neutralized lysate to a final concentration of 1M or 1% w/v with thorough mixing. Addition of extra salt had no advantage in plasmid preparations with any strains studied. Combinations of salting time (i.e. time in the presence of salt) and temperature were tried with various strains, including: 37°C for 20 to 90 min; 52°C for 60 to 90 min; 52°C for 25 min plus 22°C for 20 min; room temperature (22°C) for 30 min; and 0°C for 30 min. It was found that high temperatures (52°C) or low temperatures (0°C) were poor conditions to use, since they led to smeared plamid profiles or to low plasmid recovery. Without exhaustive testing, it was determined that the best isolation was achieved with room temperature incubation for 30 min.

The effect of phenol extraction was apparently dependent upon 2 major factors: the degree of equilibration of the phenol with 50/20/1000 TES prior to extraction; and the thoroughness of mixing of the 2 phases and subsequent separation. It was found repeatedly that un-equilibrated or poorly equilibrated phenol did not allow for optimum recovery of plasmids. Rather, the plasmids remained either in the water-phenol interfacial material or were partitioned into the phenol phase. This plasmid partitioning into the interface under inadequate ionic conditions was also noted by Hogrefe and Friedrich (1984). To remedy this, "back-extractions" of the phenol and interfacial material with fresh TES was carried out. Preparations which did not show plasmids in the first aqueous phase would demonstrate plasmids in the "back-extracted" TES phase. As long as the equilibration of phenol and subsequent mixing of the phases were adequate, "back-extraction" did not reveal a significant amount of plasmid material, and could be dismissed. These findings seem to confirm that the plasmids are indeed membrane-associated and probably also chromosome-associated.

The quality of the phenol used also had an effect on the plasmid preparation. It was noted that liquefied phenol (90%, Folin-Ciocalteau reagent; Fisher) was adequate for use as long as it was completely colorless. The presence of even a slight straw color (indicative of photolysis products) decreased plasmid isolation. The use of crystalline phenol, melted immediately prior to use, and equilibrated with TES, seemed to improve plasmid preparation. In addition, storage of the phenol at -20°C between uses seemed to allow for more consistent isolations. However, the use of ultrapure phenol (BRL) had no apparent positive effects on preparations; the pure crystalline phenol (BDH or Mallinckrodt) was adequate, contrary to C.I.Kado (personal communication, 1983). Elimination of the phenol extraction step was detrimental to plasmid isolation.

Some published procedures include chloroform in the phenol-extraction step, intending to effect more complete phase separation after extraction. Inclusion of a $CHCl_3$ extraction step had little effect on plasmid preparation from *Flavobacterium* (8W-2).

The duration of ethanol precipitation at -20°C had an effect on plasmid recovery, as studied with *Flavobacterium* (8W-2) plasmids. Incubation at -20°C for 17 hr or less led to an inferior plasmid preparation; incubation for 23 to 42 hr was optimal. Between 42 and 72 hr, the quality of the plasmids recovered decreased. Therefore, as a standard procedure, the plasmids were ethanol precipitated at -20°C for a minimum of 18 hr, and usually for 24 hr.

Dissolution of the precipitated plasmids after recovery seemed to be important with concentrated plasmid preparations from both laboratory and environmental strains. That is, inadequate volumes of dissolving buffer produced extremely viscous samples which would not load on the gel properly, and which displayed smeared bands, or showed excessive chromosomal material which obscured the plasmid bands. There seemed to be little difference between distilled water, 10/4 TE and 50/20 TE as solvents. There was no apparent difference between the use of an SDS/glycerol-based and a Ficoll-based loading buffer regarding plasmid quality. As a precaution against contamination, the diluents were freshly prepared from refrigerated stock reagents, and the loading buffers were stored at -20°C. Regarding electrophoresis, the vertical gel apparatus designed by K.Roy (Dept. Microbiology, University of Alberta) was superior to commercial horizontal gel apparatus (Bethesda Research Laboratories; or Tyler Products), producing sharper bands and straighter lanes in less time. Several vertical gel boxes with different dimensions were constructed, allowing for flexibility in choosing the appropriate size gel for particular applications.

3.3.2 Summary

Most of the techniques reported here were suitable (without modification or with minor changes) for isolating plasmids from laboratory or reference strains, but the use of environmental isolates required optimization of the techniques. In particular, isolation of the plasmids from *Flavobacterium* (8W-2) demanded extensive modification of published procedures.

The difficulties encountered with attempted isolation of *Flavobacterium* (8W-2) plasmids were not unique to this study. Many laboratories studying bacterial species other than *E. coli*, or dealing with large plasmids in particular, have experienced difficulties. The elusiveness of the plasmids may be technical in nature, or may be attributed to inherent genetic problems of the host bacterial strain, or to a combination of these effects (Singer and Finnerty, 1984).

For example, while alkaline lysis proved to be more effective than neutral lysis for most large plasmids (Mickel *et al.*, 1977), Rosenberg *et al.* (1982) encountered technical difficulties isolating "megaplasmid" DNA even with controlled pH lysis. Hogrefe and Friedrich (1984) found that consistent isolation of large plasmids was dependent upon the optimization of a number of technical chemical and physical factors. Conversely, Conners and Barnsley (1982) found a biochemical/genetic basis for inconsistent plasmid isolation when they attempted the isolation of NAH plasmids from a *Pseudomonas* strain by several published methods and found each to be unsatisfactory. Eventually they had to transfer the plasmids to another *Pseudomonas* strain which allowed reproducible isolation of NAH. Similarly, the *Pseudomonas* strain PAO has been reported to contain cryptic plasmids which are isolated only inconsistently with current methods. Strom *et al.* (1984) have reported that, even with strains of the same species, lysis and enrichment techniques may have to be optimized to achieve consistent plasmid isolation. The biochemical basis for poor isolation has not been explained.

It is probably a combination of these reasons (technical and genetic) which precluded easy isolation of plasmids from *Flavobacterium* (8W-2). However, using appropriate precautions, routine isolation of plasmids from the *Flavobacterium* isolate was eventually accomplished. Fortunately, plasmid purification with the other two isolates was relatively easy.

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Chapter 4

Characterization of Phenotypic Variants

4.1 Introduction

A technique commonly used to relate plasmids to specific functions is to "cure" the strain of the plasmid(s) and observe the resulting phenotypic changes. This method of inferring the presence and function(s) of degradative plasmids was reported in initial studies (Chakrabarty, 1974) before the plasmids were ever isolated. That is, strains of *Pseudomonas putida* capable of toluene or naphthalene degradation were subjected to common curing procedures. When phenotypic variants (e.g. TOL$^-$ or NAH$^-$) arose and failed to spontaneously revert with a measureable frequency, or were transferred to recipients by conjugation, it was assumed that the degradative genes were located on an extrachromosomal element (Chakrabarty, 1972; Chakrabarty *et al.*, 1973; Dunn and Gunsalus, 1973). It was only later that the actual TOL and NAH plasmids were isolated as physical evidence for the existence of the proposed degradative plasmids.

It has since been found that expression and regulation of the TOL and NAH phenotypes are not simple genetic phenomena, since in some organisms there is chromosomal involvement in aromatic degradation (Zuniga *et al.*, 1981; Haas, 1983), and certain deletions in degradative plasmids may produce phenotypes indistinguishable from cured isolates

(Cane and Williams, 1982). However, the original observation that curing agents caused phenotypic changes was exploited in this project.

The process of curing interferes with maintenance of plasmids in the population. This can be accomplished in several manners by chemically interfering with replication of plasmids, or by inducing the chromosomal DNA in the population to replicate faster than the plasmid DNA, thus diluting the plasmid content of the population. Since chemical curing agents may induce many different, non-specific DNA disruptions, treatment with curing agents may result in deletions, insertions, point mutations, etc., rather than (or in addition to) plasmid removal. Therefore, phenotypic variants must be sorted into truly cured strains and genetically altered strains.

In this study, a variety of curing techniques were used in an attempt to completely cure the isolates of their plasmids, or, failing that, to generate genotypic variants to study. Screening of the isolates was carried out in a variety of ways to test several different phenotypes. A large number of phenotypic variants were isolated in an attempt to correlate phenotype with genotype, and thereby to correlate plasmids with PAH degradative capacity.

4.2 Methods and Materials

4.2.1 Curing Techniques for Generating Phenotypic Variants

Growth at high temperature (Stadler and Adelberg, 1972), a "physical" curing method, was used with *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1). The isolates were inoculated into STCS and incubated with shaking at 30, 37, 44.5 or 46°C. The flask showing the most rapid increase in turbidity at the highest temperature was diluted and plated onto BMA or PCA for replica plating.

Alternatively, the isolates were grown in successive transfers of rich broth (STCS) without selective pressure at normal incubation temperatures (Furukawa and Chakrabarty, 1982; Kiyohara *et al.*, 1982a). Aliquots of each transfer were plated onto BMA for replica plating to look for spontaneous loss of plasmids.

Growth on benzoate-minimal medium was reported to be very successful in isolating TOL[illegible] strains of *P. putida*, due to a regulatory quirk of benzoate metabolism whereby cured cells utilizing benzoate by *ortho*-cleavage grow faster than TOL-containing cells using *meta*-cleavage (Chapter 1). Benzoate-curing of the *Flavobacterium* (8W-2) and *P. putida* (ATCC 33015) cultures was attempted by inoculation into a minimal medium (ASW or Jobson's) with 5mM benzoate as a carbon source. Successive transfers of *P. putida* (ATCC 33015) were carried out daily for 14 days, and aliquots were plated onto PCA for subsequent replica plating onto minimal agar with m-toluate as sole carbon source.

Similarly, *Flavobacterium* (8W-2) was maintained by 4-day transfers at 26°C in ASW + 5mM o-phthalate (Kiyohara *et al.*, 1981), the phenanthrene pathway equivalent of the toluene-degradation intermediate, benzoate. After 7 transfers, aliquots were plated onto BMA for replica plating to screen for phenotypic variants which had spontaneously lost plasmids.

The following chemical curing agents were employed in attempts to cure the three isolates being studied: acriflavin HCl, mitomycin-c, ethidium bromide and novobiocin. Stock filter-sterilized solutions were added in varying concentrations to either basal (ASW + NP + 1% succinate) or rich (STCS) broth and inoculated with *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) or *Aeromonas* (38W-HC10-1). The tube with the highest concentration of curing agent which still showed growth (i.e. sublethal concentration) was plated onto BMA or PCA for subsequent replica plating. Alternatively, aliquots of the cultures from sublethal concentrations were transferred to rich broth for "recovery" in the absence of selective pressures, then plated. The concentrations of curing agents needed to effect sublethal conditions varied widely depending upon the agent and the isolate.

In some experiments, the surface "gums" of *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) were removed enzymatically prior to exposure to curing agents to make the isolates more sensitive to the curing agents. The method of Negoro *et al.* (1980) was modified as follows. Ten milliltres of 48 hr STCS cultures were harvested and washed with sterile ASW + 0.01% YE. The washed pellet was resuspended in 5mL of ASW + 0.01% YE containing approximately 3mg each of Pronase® (Sigma) and Lytic Enzyme L1 (from *Cytophaga*; BDH) and incubated at 37°C for 90 min. The cells were washed twice in ASW + 0.01% YE. One drop of resuspended cell pellet was used to inoculate tubes containing curing agents in 5mL of basal or rich medium.

4.2.2 Replica Plating

Replica plating was carried out on different media to test various phenotypes. A sterile toothpick was used to transfer part of an isolated colony to a master plate (BMA) and selective or differential plates. These plates included: BMA plates containing 10µg $HgCl_2$/mL; 100µg $CdCl_2$/mL; 40mg streptomycin sulfate/mL, or sprayed by the method of Kiyohara *et al.* (1982b) with biphenyl, naphthalene, phenanthrene, dibenzothiophene, or fluorene. The antibiotic- and metal-containing plates were scored for growth, while the hydrocarbon-containing plates were scored for production of colored intermediates and/or cleared zones around colony growth. Combinations of selective and differential plates were used, and were compared with master plates. Phenotypic variants were re-isolated from the corresponding master plates.

Cultures of the control *P. putida* (ATCC 33015) from benzoate-containing medium were plated on PCA and replica plated to PCA master plates and selective plates of minimal medium + 5mM m-toluate. Growth on the m-toluate minimal medium plates was compared to the master plate. Those isolates incapable of growth on m-toluate (m-Tol$^-$) were scored as variants and re-isolated onto PCA.

Since the toothpick method of replica plating was tedious when screening thousands of colonies for phenotypic variation, a velvet block replication method was tried. Sterile velveteen squares were stretched over an aluminum block and held in place with a metal clip. Cultures from curing studies were diluted and plated onto BMA. After incubation, plates displaying well-separated colonies were replica plated by pressing the sterile velveteen onto the plate, then inoculating subsequent selective and differential plates with the velveteen-covered block (Smibert and Kreig, 1981).

4.2.3 Characterization of Phenotypic Variants

Once phenotypic variants had been defined by isolation on selective or differential plates described above, a number of tests were applied in an attempt to characterize the variants.

Quantitative estimates of the proportion of a culture displaying antibiotic and/or metal resistance were made by plating dilutions of overnight cultures onto BMA and Sm

(40µg/mL), $HgCl_2$ (10µg/mL) or $CdCl_2$ (100µg/mL) selective plates. The number of colonies appearing on the selective plates was divided by the total number of colonies appearing on the master plate to provide a "percent resistant" value.

To determine the range of utilizable hydrocarbons for variants, the Kiyohara *et al*. (1982b) hydrocarbon sprayplate technique was used in conjunction with minimal agar plates as a means of supplying a carbon source. Variants were inoculated to replicate minimal agar plates, and sprayed with single hydrocarbons (eg. naphthalene, phenanthrene, anthracene, dibenzothiophene; benzene and toluene were provided as vapors via saturated filter paper in glass petri plates). The plates were sealed with Parafilm®, incubated at 26°C in the dark, and scored for growth and/or color production compared with an inoculated unsprayed control plate.

To determine the abilities of phenotypic variants to initiate attack of PAH and to metabolize pathway intermediates (Chapter 1), three main techniques were used.

1. Initial attack on aromatic rings usually proceeds via hydroxylation. Reaction of primary aromatic hydroxyls and dihydrodiols with tetraazotized o-dianisidine can be measured spectrophotometrically. The procedure of Wackett and Gibson (1983) was modified as follows. Variants were grown in tubes of ASW + 0.01% YE, containing 0.5 to 5mM hydrocarbon substrate (i.e. naphthalene, phenanthrene, dibenzothiophene, biphenyl).

One-tenth millilitre (421 nmoles) of freshly hydrated tetraazotized o-dianisidine (2g/mL stock solution; Sigma) was added to 1mL of culture and incubated at room temperature at least 3 min. Debris in the reaction mixture was pelleted in a microfuge for 2 min, and the absorbance of the supernatant was read at 618 nm on a Pye-Unicam double-beam spectrophotometer. The blank was a reacted, uninoculated control containing the appropriate substrate. Reacted values from blanks were subtracted from the test values for a net absorbance reading.

2. Color production by some variants when grown in the presence of certain hydrocarbons and intermediates was qualitatively recorded. Growth was usually initiated in tubes of ASW + 0.01% YE containing 1 to 5mM substrate. Substrates included phenanthrene, naphthalene, and some degradation pathway intermediates: naphthoic acid, 2,3-dihydroxy-naphthoic acid, 1,2-dihydroxy-naphthoic acid, 1-hydroxy-2-naphthoic acid, protocatechuic acid, o-phthalic acid, salicylic acid, catechol, benzoic acid. (N.B. These organic acids are hereafter referred to as their salts.) The presence or absence of growth (i.e. turbidity) and production of color compared with an uninoculated control were recorded.
3. Quantitative measurement of this color production was achieved spectrophotometrically by incubating variants as above. Periodically, 1mL aliquots were removed,

sedimented for 2 min in a microfuge to remove debris, and scanned spectrophotometrically from 600 to 200 nm, blanked against uninoculated medium. The absorption spectra were compared with spectra of authentic standards dissolved in sterile culture medium in an attempt to identify accumulated pathway intermediates.

Radiometric determination of mineralization rates was accomplished using the method of Fedorak *et al*. (1982). Variants of *Flavobacterium* (8W-2) were grown in STCS at 26°C, harvested in sterile centrifuge bottles and resuspended in sterile ASW + 5% TCS broth to an equal optical density at 600 nm. Twenty millilitres of the standardized inoculum were introduced to 200mL of ASW + 5% TCS medium containing 0.2mL of PBa "spiked" with approximately 600,000 dpm of (9-^{14}C)-phenanthrene. Uninoculated flasks were included as volatility controls and replenishment sources. All flasks were sealed and incubated at 26°C with shaking in the dark. The release of $^{14}CO_2$ was monitored periodically by removing 5mL of liquid and 10mL of headspace gas from the flasks, acidifying, and flushing as per Fedorak *et al*. (1982). Liquid and headspace gas were replenished from parallel uninoculated controls. Growth of the variants was measured by recording the optical density at 600 nm.

The plasmid preparation techniques used for determining the plasmid content of phenotypic variants were generally limited to the modified Casse procedure for the three chosen isolates and their variants, and the Zuniga *et al*. (1981) procedure for m-Tol⁻ variants of *P. putida* (ATCC 33015). The procedure of Kado and Liu (1981) had limited application in screening *Aeromonas* (38W-HC10-1) variants; results from this procedure were always compared with results from the Casse procedure. In plasmid visualization, the TEAS buffer system was used for *Acinetobacter* (8W-1) and the TEB system was used for all other isolates. Characterization of the plasmids is discussed in Chapter 5.

4.3 Results and Discussion

4.3.1 Initial Screening Criteria

Initial screening for phenotypic variants was performed on selective media containing antibiotics or heavy metals, or on differential media sprayed with hydrocarbons.

The selective plates were used for indirect screening purposes. That is, resistance to antibiotics and heavy metals is often encoded on plasmids (Hardy, 1981; Silver and Misra, 1984), and Shapiro *et al*. (1980) report that combination plasmids encoding both antibiotic resistance and hydrocarbon degradation are "easy to isolate". Since *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) were resistant to some antibiotics and metals (Chapter 2), it was expected that some of these resistance markers would reside on one or more of the plasmids. Thus, loss of plasmids by curing would then be reflected in the appearance of sensitivity to specific antibiotics or metals. Admittedly, this was an indirect method of seeking isolates with altered PAH-degradation profiles, but it provided a very rapid and simple screen for variants.

The differential plates (i.e. Kiyohara sprayplates) were used for direct screening for PAH phenotypic differences. Some problems encountered with sprayplates were briefly outlined in Chapter 2. For example, the amount of PAH delivered to replicate plates varied uncontrollably. Often an isolate would show colored products on one sprayplate, but a much less pronounced phenotype on another plate with a different amount of the same PAH substrate. Also, since the colored products are degradation intermediates , their accumulation could be misleading. That is, an isolate which degraded the substrate completely and efficiently without accumulation of intermediates might show transient or very little color production; an indistinguishably similar phenotype might be produced by a cured or genetically altered isolate which could not initiate attack, or accumulated a non-chromogenic intermediate. A variant with an enzymic blockage after a colored compound would accumulate the colored intermediate, and, depending on the intensity of the chromogenic product, might be scored as a wild type rather than as a variant. As proposed by Shiaris and Cooney (1983), the occurrence of "clearing" of the opaque PAH layer on the sprayplates would seem to be a better indication of phenotype, but this was hard to assess and also was dependent on the amount of hydrocarbon delivered to the sprayplate.

The use of velveteen replication blocks was inappropriate for screening by the sprayplate method. The velveteen smeared the colonies on the replicated plates so that colonies were insufficiently separated to allow observation of either individual color production or clearing. Much better observations could be made using the tedious toothpick method where the size and separation of the isolates were controlled.

The initial screens used to detect phenotypic variants were incomplete, indirect and inadequate. A better screen would have been to incubate all curing treatment survivors with PBa to look for PAH degradation changes by GC, but this was completely unreasonable in terms of time alone, when dealing with thousands of potential variants. As well, variants blocked in later steps of degradative pathways would have been missed. Another approach would have been to screen each potential variant for plasmid content, then determine the phenotype for each genetic variant. This might have been considered were it not for the fact that none of the rapid plasmid screening procedures tested were successful with *Flavobacterium* (BW-2) (Chapter 3). The only reliable procedure was the modified Casse method, which took 2 days to complete; a maximum of 8 isolates could be screened per day. This approach was unreasonable for screening thousands of isolates. As well, variants with small deletions or point mutations would have been overlooked.

Had some definite markers on the plasmids been known, the task of screening could have been simplified tremendously, but since the original isolates were environmental, and therefore genetically uncharacterized, the plasmids were by definition cryptic. Therefore, the best that could be done was to screen potential variants for a combination of phenotypes in the most efficient manner possible, i.e. on antibiotic- and metal-containing agar, and PAH sprayplates. It is unfortunate that some of these phenotypes ultimately appeared to be unrelated to the plasmids, as will be discussed.

4.3.2 Preliminary Screening Results from Curing Experiments

The data in Table 4.1 show the preliminary results from selected curing experiments. Only those variants selected for further characterization experiments are identified with numbers. Other experiments are not tabulated because they produced no variants. Approximately 8,000 colonies of *Flavobacterium* (8W-2) were screened for phenotypic variation; more than 5,000 colonies of *Aeromonas* (38W-HC10-1) and approximately 600 colonies of *Acinetobacter* (8W-1) were screened. Survival rates were calculated for a limited number of curing experiments, and ranged from 100% to 0.00001% (data not presented). Some experiments were carried out so that a rich medium (TCS or STCS) was used as a

Table 4.1 Summary of curing experiments and list of phenotypic variants.

Culture	Curing Agent and Medium[1]	Selection[2]	# Variants / # Tested	Variant Designation[3]
Acinetobacter (8W-1)	acriflavin HCl 10μM, a	Bph 4mBph	3/100	A3
	" 50μM, a	Bph 4mBph	2/50	A6
	" 50μM, a	Bph	4/150	9
	ethidium bromide 200μM, a	Bph	2/150	
	novobiocin 10μg/mL, a	Bph	1/150	40
Flavobacterium (8W-2)	spontaneous, a	Dbt Bph	1/50	46
	" , a	Cd	4/50	B, B-1L
	" , b	Cd Sm Dbt Bph	8/260	pv80, Maint
	" , b	Hg Sm Dbt Bph	2/260	
	" , b	Cd Hg Sm	76/364	pv64
	" , c	Dbt Bph Phn	2/100	
	acriflavin HCl 50μM, a	Cd Hg Sm	59/416	
	" 50μM, a	Cd Hg	1/100	
	high temperature, a	Cd Sm Dbt	8/500	T101, T118
	" , a	Cd Sm Bph	12/540	
Flavobacterium (8W-2), treated[4]	acriflavin HCl 25μM, a	Cd Hg Sm Dbt Bph	7/1014	
	" 25μM, d	Cd Hg Sm Dbt Bph		pv40, pv43
Aeromonas (38W-HC10-1)	spontaneous, e	Dbt Nah	10/100	
	" , f	Dbt Nah	12/100	
	mitomycin-c 2μg/mL, d	Dbt	9/137	
	" 5μg/mL, a	Hg Dbt Bph Phn Flr	2/100	
	" 5μg/mL, a	Dbt	51/364	pv42, pv45, pv52, pv53, pv54
	" 10μg/mL, a	Dbt	2/150	
	" 15μg/mL, a	Phn	1/100	4
	acriflavin HCl 50μM, a	Hg Dbt Phn	4/100	
	" 250μM, d	Dbt	2/303	
	novobiocin 75μg/mL, a	Dbt Nah	11/49	N28, N29
	" 100μg/mL, a	Dbt Nah	7/40	N32, N33, N34, N35
	" 150μg/mL, a	Dbt Nah	2/16	N38, N39
	" 200μg/mL, a	Dbt Nah	2/30	N40, N41

(continued...)

Table 4.1 (continued)

Culture	Curing Agent and Medium[1]	Selection[2]	# Variants / # Tested	Variant Designation[3]
Aeromonas (38W-HC10-1)	high temperature, a	Dbt Phn	19/100	
	" , a	Dbt	20/250	
	" , a	Hg Nb	1/250	
Aeromonas (38W-HC10-1), treated[4]	spontaneous, a	Dbt	0/1014	
	" , b	Hg Nb Dbt	0/182	
	acriflavin HCl, 200μM, a	Dbt	0/167	
Pseudomonas putida (ATCC 33015)	spontaneous, g	m-tol	4/100	
	" , h(1)	"	3/100	
	" , h(2)		5/100	
	" , h(3)		5/100	
	" , h(4)		10/100	
	" , h(7)		36/100	8, 12, 13
	" , h(13)		232/300	
	acriflavin HCl 500μM, g	Cd Nb	1/233	
E. coli DT831	mitomycin-c 2μg/mL, g	Sm	2/597	
	acriflavin HCl 200μM, g	Sm	8/128	

[1] Media used: (a) STCS; (b) ASW + PBa; (c) ASW + NP + o-phthalate; (d) ASW + 0.01% YE; (e) ASW + NP + 5mM biphenyl; (f) ASW + NP + 5mM phenanthrene; (g) TCS; (h) minimal salts + 5mM benzoate (number of transfers in medium).

[2] Selection Criteria: Cd, Hg, Sm, Nb, m-tol - growth on agar containing $CdCl_2$, $HgCl_2$, streptomycin sulfate, novobiocin or m-toluate, respectively (see text); Dbt, Bph, 4mBph, Phn, Nah, Flr - lack of color production or clearing on sprayplates with dibenzothiophene, biphenyl, 4-methylbiphenyl, phenanthrene, naphthalene or fluorene, respectively, compared with wildtype.

[3] Only those variants referred to in the text are listed.

[4] Cells enzymatically treated to remove surface "gum"; see text.

"recovery" medium for isolates after exposure to curing agents on the premise that an increased number of survivors might produce a higher frequency of variants. The expected increase was not observed (data not presented), and the use of rich "recovery" media was discontinued. The concentration of chemical curing agent required to produce sub-lethal growth conditions was strain- and agent-dependent, as found by Chakrabarty *et al.* (1973). Generally, a higher concentration of chemical curing agent was required to produce a lethal concentration in rich medium, like STCS, compared with a minimal medium like ASW + YE, since the rich medium seemed to provide a protective effect. Similarly, the surface "gum" layer of *Aeromonas* (38W-HC10-1) and *Flavobacterium* (8W-2) may have provided protection from chemical curing agents since enzymatic removal of the surface layer resulted in lower sublethal concentrations; however, stripping of the surface layer did not result in an increased frequency of variant isolation.

Chemical curing agents were generally ineffective in producing phenotypic variants from *Flavobacterium* (8W-2), and mitomycin-c was inhibitory to growth at very low concentrations (1µg/mL). Benzoate minimal medium was completely ineffective, since *Flavobacterium* (8W-2) did not grow with benzoate as sole carbon source. Growth of *Flavobacterium* (8W-2) in o-phthalate minimal medium, suggested by Kiyohara *et al.* (1981), resulted in a low level of variant production after a month of transfers (7 subcultures). This contrasts with the reports of Kiyohara *et al*. (1981, 1982a) that the frequency of curing of PHN$^+$ *Alcaligenes* AFK2 increased with the number of subcultures in o-phthalate, to a maximum of 100% after only 20 transfers. (However, neither the wild type nor the putative "cured" PHN$^-$ variants were subjected to plasmid isolation techniques in those studies.) It is possible that extended transfer of *Flavobacterium* (8W-2) in o-phthalate would have resulted in an increased incidence of phenotypic variants. However, Ghosh and Mishra (1983) reported that, contrary to Kiyohara *et al*. (1982a), a phenanthrene-degrading *Micrococcus* sp. was not cured by growth in o-phthalate.

Spontaneous curing by transfer in rich medium and by growth in rich medium at high temperature were effective in generating variants of *Flavobacterium* (8W-2) as suggested by other workers: spontaneous curing also was noted by Furukawa and Chakrabarty (1982) during growth of biphenyl-degrading bacteria in rich medium, and Shields *et al*. (1983) found that 4-chloro-biphenyl-degrading environmental isolates were insensitive to all curing methods except growth at high temperature. "Spontaneously cured" isolates of *Flavobacterium* (8W-2) were chosen for study by picking colonies of different size or color from plates of old maintenance cultures or from STCS transfers. Colonies of different size were chosen because of observations regarding the size of cured (TOL$^-$ or SAL$^-$) strains of *Pseudomonas*. Williams and Murray (1974) observed that TOL$^-$ strains of *P. putida* grown on benzoate minimal agar grew faster and therefore to a larger size than TOL⁺ strains because the chromosome-encoded ortho-cleavage of catechol is a more efficient pathway than the plasmid-encoded meta-cleavage pathway. Similarly, Austen and Dunn (1980) observed that large colonies of certain *Pseudomonas* spp. grown on benzoate and phenol plates were found to be regulatory mutants of the meta-cleavage pathway encoded on plasmids pND140 and pND150. Therefore, the largest colonies of *Flavobacterium* (8W-2) were chosen from spontaneous curing plates in the hope that they would be cured or genetically altered, and several of these large isolates were shown to be phenotypic variants. Colonies showing variations in shades of yellow were chosen in the hope that a surface phenomenon like pigmentation might be reflected in metabolic alteration (eg. uptake and utilization of PAH).

A major effort was directed towards curing *Aeromonas* (38W-HC10-1), since the wild type contained only one plasmid, as opposed to *Acinetobacter* (8W-1) and *Flavobacterium* (8W-2) which contained multiple plasmids. However, the limited PAH phenotype of *Aeromonas* (38W-HC10-1) made selection of PAH variants more difficult, and the inherent problems of the sprayplate technique were encountered again. Novobiocin, as suggested by W.Finnerty (University of Georgia, personal communication, 1982; Monticello *et al.*, 1985), proved to be the most effective chemical curing agent for *Aeromonas* (38W-HC10-1), while mitomycin-c was effective with the standard *E. coli* DT831 at a low frequency. *Acinetobacter* (8W-1) was sensitive to several chemical curing agents.

P. putida (ATCC 33015) was included as a curing control and also was subjected to curing agents to produce a TOL⁻ recipient for later conjugation studies (Chapter 5). It was quite insensitive to chemical curing, as reported by Williams and Murray (1974). The most effective method for "curing" *P. putida* (ATCC 33015) was growth in benzoate minimal medium, because of the quirk of catechol metabolism regulation previously discussed. However, the m-Tol⁻ *P. putida* (ATCC 33015) variants showed a consistent decrease in plasmid size of 33 Mdal rather than elimination of the plasmid. This observation corresponded to reports by Bayley *et al.* (1977) that many m-Tol⁻ variants, phenotypically indistinguishable from TOL⁻ cured strains, are actually the result of precise, large deletions of the TOL plasmids. The deletions remove the degradative genes and leave behind the replicative portion of the plasmid. This was found to occur at a high frequency by Williams and Murray (1974). The excision remnant of the TOL plasmid was found in this study to be approximately 47 Mdal, which compares well with the value of 51 Mdal reported by Downing *et al.* (1979).

Although a number of phenotypic variants of *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) were generated in these curing experiments, no strains were ever found which were entirely free of plasmids. The phenotypic variants always showed one or more plasmids, although in most cases the patterns (i.e. numbers and/or sizes) of plasmids were different from the wild type, as will be discussed later in this chapter. Generally, phenotypic variants of *Acinetobacter* (8W-1) showed a reduction in plasmid number; the *Flavobacterium* (8W-2) variants showed great diversity in plasmid number and size; but the *Aeromonas* (38W-HC10-1) variants showed a single, large plasmid of apparently constant relative mobility, regardless of phenotype.

Although failure to isolate completely cured strains was puzzling and frustrating, other workers have also reported difficulty in curing PAH-degradative isolates. An early paper by Chakrabarty *et al.* (1973) reported that they were unable to cure naphthalene use in a strain of *P. putida* using mitomycin-c; they then surmised that the degradative genes were chromosomal, but did not present any supporting evidence. Shields *et al.* (1983) reported that chemical curing agents were not effective in eliminating plasmids from 4-chloro-biphenyl degraders. Dunn and Gunsalus (1973) were unable to cure a strain of *P. putida* PpG7 of the NAH plasmid using mitomycin-c, and Connors and Barnsley (1982) reported that NAH$^+$ *Pseudomonas* strains ATCC 17483 and 17484 could not be cured: the latter strain showed multiple plasmids, regardless of curing treatment. Masse *et al.* (1984) reported a very low frequency of variant formation in a 4-methyl-biphenyl-degrading *Achromobacter* sp. using either mitomycin-c or EtBr. Kiyohara *et al*. (1983) reported that mitomycin-c and acridine orange treatments were unsuccessful in curing the Phn$^+$ phenotype from a *Beijerinckia* sp., but that growth in benzoate minimal medium was successful. Even so, only some plasmid species were cured in Phn$^-$ Bph$^-$ strains with this method. With *Alcaligenes* AFK2, Kiyohara *et al*. (1982a) reported very low rates of curing with mitomycin-c, acridine orange, SDS, and no curing with EtBr. They also reported a 0% rate of spontaneous curing in rich medium, having tested 13,522 colonies for the Phn$^+$ phenotype. This would appear to indicate unusual stability of the proposed PHN plasmid in the absence of selective cultural pressure. Kiyohara *et al*. (1981) also reported that the AFK2 strain contained two plasmids, and mitomycin-c curing of either one correlated with the appearance of the Phn$^-$ phenotype. However, their report infers that some Phn$^-$ strains were not cured of either plasmid.

It would appear from these and other reports that some degradative plasmids are considerably less susceptible to chemical or spontaneous curing than either R-factors or F-factors. The reason for this apparent stability *in vivo* has not been discussed in the literature. It is possible that chromosomally-encoded degradative genes are responsible for the apparent perseverance of the phenotypes. Certainly, composite phenotypes for degradative pathways have been described, where part of the pathway is chromosomally encoded and part is plasmid encoded (Austen and Dunn, 1980;

Zuniga *et al*., 1981), or where duplication of degradative genes occurs. In this case, loss of the plasmid might not be reflected in the appearance of PAH$^-$ variants.

4.3.3 Phenotypic Characterization of *Acinetobacter* (8W-1) Variants

Acinetobacter (8W-1) is limited to degradation of biphenyl, 3-methyl- and 4-methyl-biphenyls (Fedorak and Westlake, 1983). Therefore, cultures were screened for variants by the sprayplate technique using biphenyl and 4-methyl-biphenyl (Table 4.2). The colors produced from Bph$^+$ and 4mBph$^+$ colonies correspond to the yellow meta-cleavage compounds described by Furukawa *et al*. (1983) and Fedorak and Westlake (1983); gradations of color production may occur. This may be a function either of the rate of cleavage or the rate of substrate degradation of the cleavage compound to non-chromogenic product(s).

The wild type did not produce much color from biphenyl, yet cleared the opaque layer of biphenyl on sprayplates. This, in conjunction with ^{14}C-biphenyl mineralization experiments (Chapter 2), indicated that extensive metabolism of biphenyl occurred in the wild type. The small amount of color observed on biphenyl sprayplates may be due to efficient degradation of the yellow meta-cleavage products to non-chromogenic compounds rather than being due to non-utilization of biphenyl. It is possible that the equally small amount of color produced by variant #12 is due to lack

Table 4.2 Metabolism of biphenyl and 4-methylbiphenyl by *Acinetobacter* (8W-1) wild type and 6 phenotypic variants. Cultures were inoculated onto BMA plates, sprayed with hydrocarbons, and incubated at 26°C in the dark.

	Acinetobacter (8W-1) Variants						
Phenotype	wt	#9	#12	#40	A1	A3	A6
Biphenyl (color)	±[1]	±	±	++	±	±	±
Biphenyl (clearing)	+	±	-	+	+	+	-
4-Methyl biphenyl (color)	+	+	+	+	±	++	+

[1] ++, +, ±, - : decreasing amounts of colored products or clearing of opaque hydrocarbon layer

of metabolism of the biphenyl, since variant #12 failed to clear the biphenyl layer. Spectrophotometric analysis of culture supernatants might have solved this question.

Since the fate of biphenyls in variant cultures was not determined, the reason for accumulation of colored compounds in variants #40 (overproduction of color from biphenyl) and A3 (overproduction of color from 4-methyl-biphenyl) is not known. At least two possible explanations must be considered.

1. The overproducers may be regulatory mutants, such that the initial biphenyl meta-cleavage enzymes are induced by growth on biphenyl (as suggested for the wild type, Chapter 2), but the enzymes for subsequent degradation of the meta-cleavage products are not induced. Alternatively, the first enzymes of the pathway might simply have escaped feedback regulation. Either of these postulated variants would accumulate colored products. This phenotype could occur regardless of the location of the regulatory and structural genes, whether completely chromosomal, completely plasmid-encoded, or as a combination of chromosomal and plasmid genes.
2. The variants may have a structural basis; that is, the genes for degradation of the colored meta-cleavage compounds may be inactive or absent (e.g. due to deletion or loss of a plasmid).

The difference between structural and regulatory mutants might be discerned by analysis of the meta-cleavage products. Constitutive levels of the later enzymes of the degradative pathway (presumably found in regulatory mutants) should eventually deplete the accumulated colored products, whereas no depletion should be observed in the structural mutants.

There is the possibility that a "composite phenotype" can be found in *Acinetobacter* (8W-1), as noted by Dunn *et al.* (1980) and Zuniga *et al.* (1981) with certain NAH plasmids, by Wong and Dunn (1976) for phenol degradation, and by Poh and Bayly (1980) for xylenol degradation. This would require that some of the regulatory or structural genes be present on the chromosome, while others would reside on one or more plasmids. The regulatory interactions of these genes could be very complex regarding induction and feedback inhibition. Thus, a Bph$^-$ phenotype might be reflected in the loss of a plasmid, or in plasmid deletions, or in no change in plasmid content in the case of a chromosomal mutation.

Very little work was done with *Acinetobacter* (8W-1) because other research groups began concentrating on biphenyl degraders during the course of this project (Furukawa *et al.*, 1983; Shields *et al.*, 1983; Sayler *et al.*, 1984). For this reason, *Acinetobacter* (8W-1) was abandoned in favor of *Flavobacterium* (8W-2), which had a wider range of degradative capability, and was therefore of more interest.

4.3.4 Phenotypic Characterization of *Aeromonas* (38W-HC10-1) Variants

Spontaneous variants of *Aeromonas* (38W-HC10-1) chosen from cultures grown with biphenyl or phenanthrene were screened for color production on dibenzothiophene or naphthalene sprayplates. A variety of phenotypes were observed: decrease in color produced from dibenzothiophene and/or naphthalene; different colors produced (eg. brown or yellow from naphthalene); lack of clearing of naphthalene; or combinations of these phenotypes. When retested in ASW + 0.01% YE plus phenanthrene or dibenzothiophene, color production by most variants correlated positively with the diverse phenotypes on sprayplates.

Screening of high temperature-generated variants of *Aeromonas* (38W-HC10-1) with dibenzothiophene sprayplates produced a number of Dbt$^-$ variants, very few of which were novobiocin sensitive (at 10μg/mL) or mercuric ion sensitive (at 10μg/mL $HgCl_2$). All variants tested for plasmid content were indistinguishable from the wild type in terms of relative mobility of the plasmid DNA.

A number of variants generated by growth in novobiocin-containing STCS broth were chosen for decreased color production on naphthalene or dibenzothiophene sprayplates. When retested in ASW + 0.01% YE with dibenzothiophene or phenanthrene as substrates, no color was produced. In case the variants were deficient in different aspects of PAH metabolism, they were tested for complementarity by incubating pairs of the variants in dibenzothiophene- or phenanthrene-containing medium, but no color production was observed. All variants were resistant to $HgCl_2$ and tetracycline (and novobiocin, by definition, having survived growth in novobiocin medium).

Selected variants from mitomycin-c and novobiocin curing studies were used in experiments studying the degradation of PAH pathway intermediates. Tubes containing 5mL of ASW + 0.01% YE plus 5mM salicylate, catechol, protocatechuate, or o-phthalate were inoculated with cell suspensions of variants and scored for color production and/or turbidity. Salicylate and catechol are intermediates of the naphthalene degradation pathway and of the proposed *Pseudomonas* phenanthrene degradation pathway, while protocatechuate and o-phthalate are intermediates of the proposed *Aeromonas* phenanthrene degradation pathway (Chapter 1; Cripps and Watkinson, 1978). Both sets of intermediates were tested because (a) *Aeromonas* (38W-HC10-1) is able to grow on naphthalene and may use the salicylate pathway, and (b) 38W-HC10-1, being an *Aeromonas* species capable of limited phenanthrene degradation, may use the protocatechuate pathway. In addition, benzoate, naphthalene, naphthoate, and 2,3-dihydroxy-naphthalene were tested with *Aeromonas* (38W-HC10-1). Selected results are presented in Table 4.3. All tabulated variants were able to produce low levels of color slowly from dibenzothiophene (compared with the wild type) and were novobiocin resistant. Culture appearances in

Table 4.3 Degradative capabilities of *Aeromonas* (38W-HC10-1) wild type and 11 phenotypic variants.

	Substrate							
Variant	Salicylic Acid	Benzoic Acid	Catechol	o-Phthalic Acid	Protocate-chuic Acid	Naptha-lene	2,3-Di-OH-Naphthalene	Naphthoic Acid
wt	+/+[1]	+/-	-/-	+/-	±/+	±/-	±/-	-/-
M42	+/+	+/+	-/-	+/-	+/-	±/-	±/-	-/-
M45	+/+	+/+	-/-	+/-	+/-	±/-	±/-	-/-
M52	+/+	+/+	-/±	+/-	+/-	±/-	±/-	-/-
M53	+/+	+/+	-/-	+/-	+/±	±/-	±/-	-/-
M54	+/+	+/+	-/-	+/-	+/±	±/-	±/-	-/-
Nb28	-/-	+/-	-/-	+/-	+/+	-/-	±/-	-/-
Nb29	-/-	+/-	±/+	+/-	-/-	-/-	+/±	-/-
Nb32	-/-	+/-	-/-	+/-	±/+	±/-	±/-	-/-
Nb33	-/-	+/-	-/-	+/-	-/-	-/-	±/-	-/-
Nb34	-/-	+/-	±/+	+/-	+/-	-/-	±/-	-/-
Nb35	±/-	+/-	-/-	+/-	-/-	-/-	+/-	-/-

[1] Growth (measured by turbidity increase)/color production (compared with uninoculated medium): + = growth or color change; ± = poor growth or slight color change; - = no growth or color change

benzoate and salicylate tubes appeared to divide the variants into two groups correlated to their mode of "curing": that is, the mitomycin-c-generated variants were all similar to the wild type in salicylate medium but dissimilar in benzoate medium, producing a yellow color; the novobiocin-generated variants were dissimilar to the wild type in salicylate medium, being unable to grow, but similar to the wild type in benzoate medium. The significance of the division is not apparent. A mixture of phenotypes was observed in protocatechuate medium. Naphthalene was able to support only limited growth of the wild type, the mitomycin-c-generated variants and one novobiocin-generated variant. From these results, it would appear that at least two degradative pathways exist in *Aeromonas* (38W-HC10-1): one via salicylate and/or benzoate, and another via o-phthalate and protocatechuate. It has been shown by Barnsley (1983) that the pathways for degradation of naphthalene via salicylate and of phenanthrene via o-phthalate are separate and regulated independently in *Pseudomonas*. This may be the case in *Aeromonas* (38W-HC10-1) also.

The observation that all 19 variants tested in o-phthalate tubes were positive for growth suggests that the genes for degradation of o-phthalate are integral, and may be chromosomally encoded or may be duplicated on the chromosome and plasmid. Similarly, growth in benzoate medium was constant even though production of the colored intermediates varied. This may also indicate the central nature of the intermediate and an integral function of benzoate degradative genes. The isolation of variants unable to grow on salicylate and protocatechuate may suggest a secondary role for these degradative enzymes.

In light of evidence that *Aeromonas* (38W-HC10-1) posesses genes for the degradation of the later metabolites of two phenanthrene pathways (via either o-phthalate or salicylate), it is curious that *Aeromonas* (38W-HC10-1) is limited to partial metabolism of phenanthrene, being unable to progress beyond the 2-ring structure (Chapter 2). One can speculate that an initial hydroxylative attack on phenanthrene is the product of a non-specific dioxygenase, while the isolate lacks the enzymes for the upper part of the pathway dealing with 3-ring cleavage or 2-ring attack. This might be substantiated by the observed lack of growth on naphthoate. The poor growth observed with naphthalene and 2,3-dihydroxy-naphthalene also may represent non-specific dioxygenase attack.

4.3.5 Phenotypic Characterization of *Flavobacterium* (8W-2) Variants

4.3.5.1 Antibiotic- and metal-resistance phenotypes

For reasons previously discussed, resistance to streptomycin (Sm), cadmium (i.e. $CdCl_2$) and/or mercuric ion (i.e. $HgCl_2$) was used as a criterion for screening *Flavobacterium* (8W-2) colonies isolated from curing experiments. A number of variants were isolated which differed from the wild type in being sensitive to Cd and/or Hg, while very few variants displayed Sm sensitivity.

Two assumptions were made when screening variants for the phenotypic characteristics described above:

1. that the resistance phenotypes were stable (i.e. would not revert to wild type at a measurable frequency)
2. that, since each variant arose from a single cell as an isolated colony, the phenotype would be uniformly inherited (i.e. 100% of the cells from the original variant colony would have the same genotype/phenotype).

To test these assumptions, a quantitative experiment was carried out by plating dilutions of three PAH phenotypic variants (T101, T118 and B-1L) plus the wild type onto selective plates and non-selective (BMA) plates. The number of colonies appearing on the selective plates was reported as a percentage of the total number of colonies from a parallel inoculum plated on non-selective media. All three variants plus the wild type had previously been characterized as resistant to Sm, Cd and Hg on replicate plates. It can be seen in Table 4.4 that all the colonies of the wild type, T118 and B-IL are Sm resistant, and most T101 colonies are Sm resistant. In fact, it may be argued that some T118 and B-1L colonies have become Sm

Table 4.4 Resistance of *Flavobacterium* (8W-2) wild type and phenotypic variants T101, T118 and B-1L to streptomycin, $HgCl_2$ and $CdCl_2$. BMA plates containing 40µg/mL streptomycin (Sm), 10µg/mL $HgCl_2$ or 100µg/mL $CdCl_2$ were inoculated in replicates of 3 with cell suspensions and incubated at 26°C for 1 week. A parallel series was inoculated onto non-selective BMA plates. Values represent the mean viable numbers obtained on selective plates expressed as a percentage of the mean viable numbers determined on BMA plates.

	% of colonies showing resistance		
Variant	Sm	$HgCl_2$	$CdCl_2$
wt	98.0	7.8	0.002
T101	78.3	23.3	0.008
T118	117.6	19.2	0.2
B-1L	108.7	39.7	0.1

dependent, since there are more colonies on the Sm plates than on the BMA plates. There is a wide variation in resistance to Hg and Cd, with the PAH phenotypic variants showing an increased percentage of resistant colonies compared with the wild type. For example, variant B-IL has five times as many Hg resistant colonies and 55 times as many Cd resistant colonies as the wild type; variant T118 has 100 times more Cd resistant colonies than the wild type.

It is puzzling that only a percentage of the colonies from a variant are resistant to Cd or Hg. However, these percentages would explain why Hg and particularly Cd resistance patterns of variants were often inconsistent in repeat trials of replica plating or showed intermediate growth on metal-containing plates. If a small inoculum was used in the replica plating (eg. if the heavy metal plate was at the end of a series of replicate plates and therefore received a small number of cells) the isolate might not show growth due to the low percentage of resistant cells. If a slightly larger inoculum was used, the resultant sparse growth would be recorded as a resistant phenotype.

Further studies were initiated to examine the stability of Cd and Hg resistance in the *Flavobacterium* variants. Four subclones were chosen from each variant plate in the preceeding quantification experiment. The subclones were re-tested for Cd and Hg resistances by streaking onto selective plates and scoring for growth, and the results are discussed below.

Those subclones chosen from streptomycin plates showed no consistent patterns of Cd or Hg resistance. Since the wild type and three variants in the preceeding experiment were essentially 100% Sm resistant, selecting Sm resistant subclones should not be selective for any particular metal resistance; rather, it would be expected to present a cross-section of metal resistance phenotypes. This expectation was borne out with the subclones, and indicates that the Sm resistant phenotype is not linked to either Cd or Hg resistance; it is probably chromosomal. In fact, it has been suggested by Owen and Holmes (1980) that antibiotic resistance in 22 clinical strains of *Flavobacterium* is chromosomally encoded. Similarly, Beschle *et al.* (1984) found that chloramphenicol resistance in a *Flavobacterium* species was chromosomally encoded, even though the strain carried a 10 Mdal plasmid. This chromosomal locus for antibiotic resistance would seem to be substantiated by the observation that Sm sensitive variants were very rare in cultures exposed to curing agents, and Sm resistance seemed to be stably and uniformly inherited.

Most subclones chosen from Hg plates remained Hg resistant upon transfer, but approximately 8% had become Hg sensitive, and 27% showed only intermediate growth on Hg plates. None of the Hg resistant subclones demonstrated definite Cd resistance, although some showed intermediate growth on Cd plates. Mercury resistance is known to be mediated by transposons and plasmids (Silver and Misra, 1984). Spontaneous excision and loss of a such a transposon from the chromosome or a plasmid might explain the appearance of Hg sensitive cells in a previously Hg resistant variant.

One hundred percent of subclones from Cd plates remained Cd resistant upon repeated transfer, and 70% of these were also Hg resistant, with 21% showing intermediate growth on Hg plates. Cadmium resistance has been demonstrated to be plasmid determined only in *Staphylococcus aureus* (Silver and Misra, 1984), resulting from specific energy-dependent efflux rather than enzymatic detoxification. However, Gram negative cells without the plasmid are often just as resistant as plasmid-bearing *S. aureus*, presumably through reduced uptake of Cd rather than efflux. The Cd resistance observed in *Flavobacterium* (8W-2) may be simply a cell surface phenomenon and might be unrelated to the PAH phenotypes. Interestingly, most Cd resistant subclones also demonstrated stable Hg resistance, which may support the idea of reduced metal uptake.

The original premise that the appearance of antibiotic- and metal-sensitive variants would be a quick screen for cured isolates was reasonable, considering that many resistances reside on plasmids. Unfortunately, it would appear that streptomycin resistance is chromosomally encoded, that mercuric ion resistance is unstable and unlinked to either Sm or Cd resistance, and that Cd resistance is not plasmid mediated, but probably is a surface-associated phenotype in *Flavobacterium* (8W-2). This apparently invalidates the use of antibiotic and/or metal resistance screens for indirect identification of cured strains of *Flavobacterium* (8W-2).

4.3.5.2 PAH phenotypes of subclones

The subclones described above subsequently were screened for PAH phenotypes by three separate transfers onto dibenzothiophene, phenanthrene, fluorene and biphenyl sprayplates. This was designed to test two parameters: correlation of PAH phenotype with metal- and antibiotic-resistance, or lack thereof; and stability/reproducibility of the PAH phenotype after repeated testing on sprayplates.

Generally, the results showed that there was no definite correlation between antibiotic- or metal-resistance and PAH phenotype in the subclones tested. Some trends were observed in the initial testing, but these were disproven in subsequent PAH phenotype determinations, partly because the PAH phenotypes which were displayed (i.e. color production and/or clearing on sprayplates) were inconsistent from one trial to another for a given subclone. It is not clear whether this variation is due to a "consensus" phenotype or due to the inherent variability of the screening procedure itself. A subclone displaying intense color production from fluorene, for example, in one trial would not produce color in a second trial. This made it difficult to correlate sprayplate-determined phenotypes with resistances.

The inconsistency of the PAH phenotype on sprayplates was demonstrated clearly with 12 subclones of variant T118. This variant was originally isolated as Bph$^-$ (i.e. no color or clearing on biphenyl sprayplates) yet 11 of 12 subclones produced a yellow color from biphenyl in another screening. In a second trial, only 1 of the 12 subclones produced a yellow color from biphenyl (corresponding to the ring cleavage product) but 8 of the 12 eventually produced a brown color (indicative of the chemical oxidation product of catechol, which is a degradation product of biphenyl). In a third trial, 7 of the 12 subclones produced a yellow color and none produced the brown color. Clearing of the biphenyl was not observed with any subclones in any trial. Similarly, all 12 subclones of T118 displayed an intense orange color on dibenzothiophene sprayplates in the first trial, but only 5 of the 12 showed any color in the second trial. In the third trial, only 1 of 12 produced an intense color from dibenzothiophene, while the remaining 11 subclones produced a pale orange color.

The PAH phenotypes of subclones from the other variants were equally variable with the exception of two sets of subclones from variant B-1L including subclone B-1L-25. The B-1L subclones chosen from Sm and Hg plates had a lighter yellow colony color on BMA plates, and were consistently Cd sensitive. They also consistently failed to produce color or clearing on any of the PAH sprayplates tested in all three trials. Those B-1L subclones which had been chosen for Cd resistance, however, did display the variable PAH phenotypes described above. It would appear then, that Hg and Sm resistant subclones of variant B-1L which are Cd sensitive have a consistent PAH$^-$ phenotype, and that the acquisition or retention of Cd resistance is correlated in 4 of 4 subclones with the acquisition of a variable PAH$^+$ phenotype. The significance of this observation is unknown.

It should be noted that, although sprayplate-determined phenotypes were variable, the phenotypes determined by growth of isolated colonies in liquid media (discussed in Section 4.3.6) were reproducible. This may be due to technical reasons, or because the liquid cultures displayed a "consensus" phenotype of the progeny cells, rather than the phenotype of a single colony.

Transmission electron microscopy was used to observe the cell surface of *Flavobacterium* (8W-2) variants, on the premise that structural modifications to the cell envelope might alter PAH metabolism through altered PAH uptake. A transmission electron micrograph (Plate 4.1) comparing washed cells of the wild type *Flavobacterium* (8W-2) and variant #69 shows that the wild type retained a surface layer of diffuse material, while variant #69 displayed a clean cell surface. Since it is not known whether this cell surface material is the same as the strands of hydrophobic material sloughed by the wild type under certain growth conditions (Chapter 2), the significance of its absence in variant #69 cannot be established. However, it seems likely that some phenotypic variants could result from alterations in cell surface material, or possibly in structural or transport protein changes, as suggested by Bayly and Barbour (1984) and Shapiro *et al*. (1980), rather than solely from metabolic enzyme mutations.

4.3.6 Degradative Properties of Some *Flavobacterium* (8W-2) Variants

Minimal salts plates sprayed with single hydrocarbons were used to determine the range of aromatics which would serve as sole carbon source for *Flavobacterium* (8W-2) and variants. Results are summarized in Table 4.5. It can be

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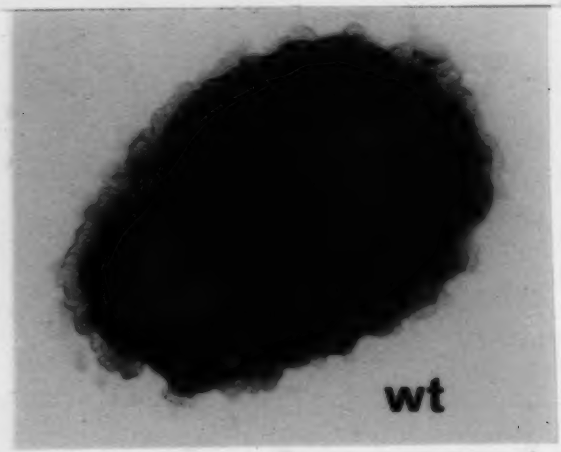

Plate 4.1 Transmission electron micrographs of *Flavobacterium* (8W-2) wild type and phenotypic variant #69 showing surface layer. Cells washed with water and stained with 2% phosphotungstic acid.

Table 4.5 Growth of *Flavobacterium* (BW-2) wild type and 7 phenotypic variants with pure hydrocarbon as sole carbon source. Inoculated minimal agar plates were sprayed with hydrocarbons and incubated at 26°C in the dark for 1 month.

	Carbon Source					
Variant	Naphthalene	Phenanthrene	Dibenzothiophene	Anthracene	Benzene	Toluene
wt	++	++,cl	col	col,cl	-	-
Maint	+	++,cl	col	cl	-	-
B-1L	-	-	-	-	-	-
T118	++	+,cl	col	col,cl	-	-
pv40	+	+,cl	col	-	-	-
pv43	+	+,cl	col	-	-	-
pv64	±	±	col	-	-	-
pv80	+	+,cl	col	-	-	-

+ = growth
++ = good growth
- = no growth or color
col= colored compounds produced
cl= clearing of opaque hydrocarbon layer around colonies

seen that naphthalene and phenanthrene serve as carbon sources for all variants except B-1L. Colored products of dibenzothiophene ring fission are produced from all variants except B-1L, but no growth was observed with this carbon source. This evidence for cometabolism of dibenzothiophene agrees with reports by Finnerty *et al*. (1983) and Hou and Laskin (1976) that isolates capable of cleaving dibenzothiophene were unable to use the compound as sole carbon source. Anthracene was partially metabolized by the wild type and variant T118, as evidenced by the production of a small amount of color, and must have been removed or solubilized by these cultures because of the clearing of the opaque hydrocarbon layer. However, no growth was observed with this substrate. No metabolism of benzene or toluene vapors was observed and no growth occurred.

Phenanthrene

The ability of two variants, #46 and B, to mineralize internally labelled (9-^{14}C)-phenanthrene was examined by the method of Fedorak *et al*. (1982). Growth was monitored as increased turbidity at 600 nm. Figure 4.1 shows that variant #46 grew only slightly more quickly than variant B, but rapidly mineralized the (9-^{14}C)-phenanthrene, releasing almost 60% of the label as $^{14}CO_2$. Variant B, however, released only 3.7% of the label as $^{14}CO_2$, indicating that it is incapable of extensive degradation of (9-^{14}C)-phenanthrene. If variant B is unable to degrade other aromatics in

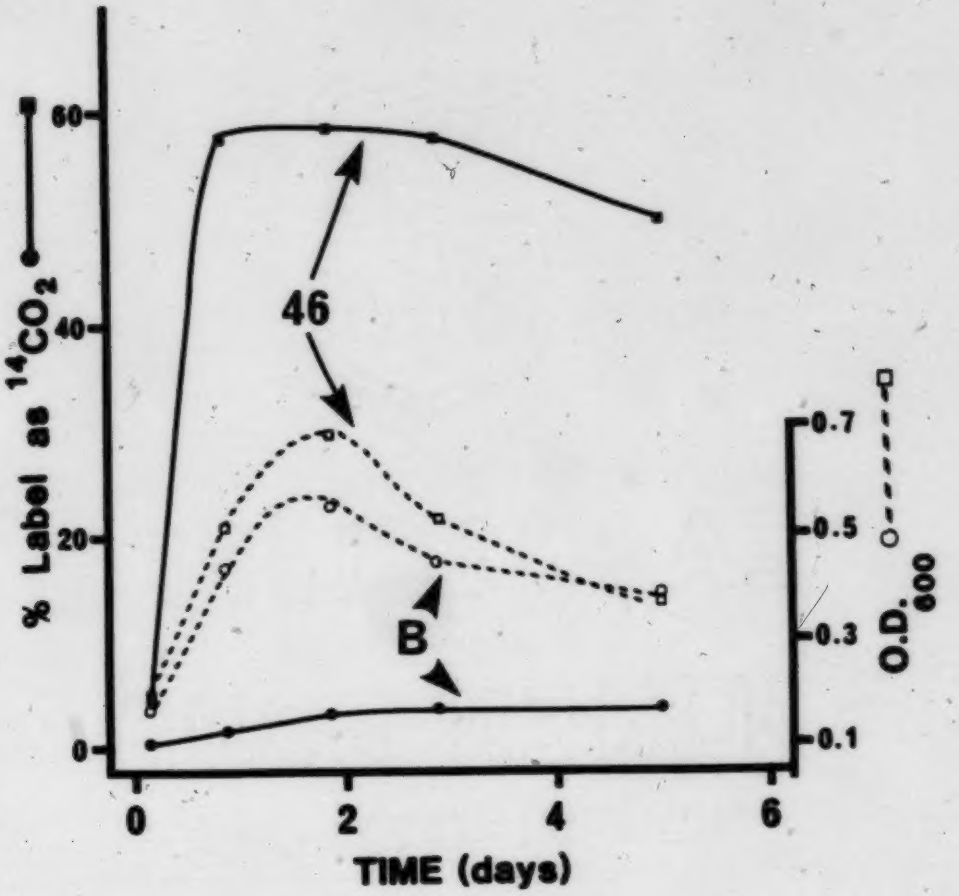

Figure 4.1 Mineralization of (9-^{14}C)-phenanthrene by *Flavobacterium* (8W-2) phenotypic variants #46 and B. Graph of culture turbidity (broken lines) and $^{14}CO_2$ evolution (solid lines) versus incubation time at 26°C in ASW + 5% STCS + PBa with (9-^{14}C)-phenanthrene.

the PBa supplement, presumably it is growing at the expense of the TCS supplement in the medium. The slightly enhanced growth of variant #46 may be attributed to utilization of the aromatics fraction in addition to the TCS supplement. In other experiments with phenanthrene, variant #46 and the wild type were shown to have virtually identical $^{14}CO_2$ release patterns and growth curves, and, in a repeat experiment, variant B was shown to release only 1.8% of the (9-^{14}C)-phenanthrene as $^{14}CO_2$.

In another radiometric experiment, the method of Westlake and Cook (1980) was used to monitor release of $^{14}CO_2$ from (9-^{14}C)-phenanthrene. The *Flavobacterium* (8W-2) wild type and variants T118 and T101 were incubated in ASW + 0.01% YE plus 5mM phenanthrene spiked with (9-^{14}C)-phenanthrene. Rather than a time course study, only a single incubation time was used. The wild type released 18.6% of the label as $^{14}CO_2$, variant T101 released 13.8% and variant T118 released only 1.1% of the label as $^{14}CO_2$ in 7 d at 26°C. The yields are considerably lower than in experiments discussed above, probably because of less growth in the absence of TCS supplement in the medium. However, it is apparent that variant T118 is not capable of extensive mineralization of (9-^{14}C)-phenanthrene.

Dibenzothiophene

Liquid cultures containing hydrocarbons were used to spectrophotometrically observe the appearance of PAH metabolites. At intervals, spectrophotometric scans were performed on cultures containing ASW + 0.01% YE plus 0.5mM to 5mM PAH. Dibenzothiophene was chosen for initial studies because it has a published degradation pathway with a defined endproduct. Because of the intensity of color produced by variant #46 from dibenzothiophene, this culture was used in initial trials. The graph in Figure 4.2 shows sequential spectrophotometric scans of a culture of *Flavobacterium* (8W-2) variant #46 grown in the presence of 2.5mM dibenzothiophene. Authentic intermediates of the dibenzothiophene degradation pathway were not available commercially to aid in identification of spectrophotometric peaks. Therefore, the literature values for absorption maxima presented in Table 4.6 were used. Comparison of the scans in Figure 4.2 with reported literature values for degradative intermediates indicated that *Flavobacterium* (8W-2) followed the dibenzothiophene degradation pathway reported by Laborde and Gibson (1977), Monticello *et al.* (1985) and Kodama *et al.* (1970). Within 10 hr, variant #46 accumulated the dibenzothiophene ring fission product [*trans*-4(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoate; D2] without accumulation of the hydroxylated intermediate [*cis*-(1,2-dihydroxy-1,2-dihydrodibenzothiophene; D1]. By 22 hr both the hydroxylated ring structure (D1) and the final product (3-hydroxy-2-formyl-benzothiophene; D3) had accumulated. Prolonged incubation lead to a decrease in the amount of ring fission product (D2) with further

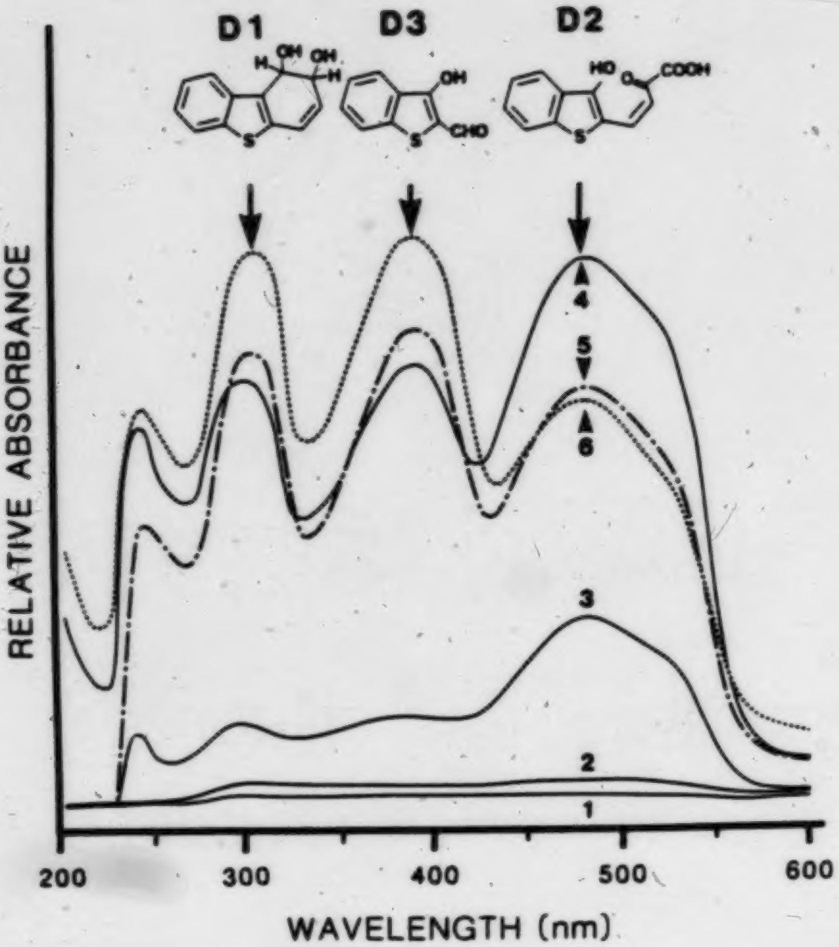

Figure 4.2 Spectrophotometric scan of culture supernatants from *Flavobacterium* (8W-2) phenotypic variant #46 incubated with dibenzothiophene. Culture grown in ASW + 0.01% YE + NP plus 2.5mM dibenzothiophene at 26°C in the dark. Samples removed at intervals, clarified by centrifugation, and blanked versus parallel uninoculated medium. Trace 1: 2hr; Trace 2: 5 hr; Trace 3: 10 hr; Trace 4: 22 hr; Trace 5: 34 hr; Trace 6: 50 hr incubation.

Table 4.6 Absorption maxima of authentic standards, and reported values for some PAH degradation intermediates.

PAH	Degradation Intermediates	Absorption Maxima (nm)	References'
Dibenzothiophene	1,2-dihydroxy-dihydrodibenzothiophene (D1)	215,265 305,315	8,9
	trans-4(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoic acid (D2)	475	5,7,8
	3-hydroxy-2-formylbenzothiophene (D3)	390	7,8
Phenanthrene	1-hydroxy-2-naphthoic acid (P2)	254,288,298 332,340	6,10
	o-phthalate (P3)	225,275	2,6,10
	protocatechuate (P4)	259,293	2,10
Naphthalene	1,2-dihydroxynaphthalene	254,338	10
	salicylate	209,230,296	1,10
	catechol	214,270 274,280	2,10
Biphenyl	yellow *meta*-cleavage compound	434	3,4
	benzoate	227 246,260,266	2 10

'References: (1) Cane and Williams (1982); (2) CRC Handbook (1978); (3) Fedorak and Westlake (1983); (4) Furukawa *et al.* (1983); (5) Hou and Laskin (1976); (6) Kiyohara *et al.* (1976); (7) Kodama *et al.* (1973); (8) Laborde and Gibson (1977); (9) Monticello *et al.* (1985); (10) this study, determined from authentic standards accumulation of both the hydroxylated ring structure (D1) and the final product (D3). The sequence of appearance of the pathway intermediates provided information regarding the regulation of the pathway. Initial accumulation of the fission product (D2) would indicate that the hydroxylation and ring cleavage proceed rapidly, and may be inducible. The enzymes which degrade the ring fission compound (D2) appeared to be induced only after a certain amount of the cleavage product (D3) had accumulated. After the final product (D3) had begun to accumulate, the hydroxylated intermediate (D1) also began to collect without an increase in the cleavage product (D2). The simplest explanation for these observations was to propose two types of control, as represented in Figure 4.3. The first enzymes of the pathway may be inducible or constitutive; this experiment cannot distinguish between them. Degradation of the ring cleavage product (D2) must be stimulated by the ring cleavage product itself, while the final degradation product (D3) would exert a feedback control at the level of the ring cleavage enzyme. This scheme would explain why variant #46 was originally isolated as an overproducer of color on dibenzothiophene, since both the colored ring cleavage and final products must accumulate before degradation is stopped. The first part of this regulatory scheme may be analagous to the regulation of the *ortho* cleavage of catechol, as reported by Cain (1980). In that *Pseudomonas* species, high levels of the dihydroxylated substrate (catechol) had to accumulate before

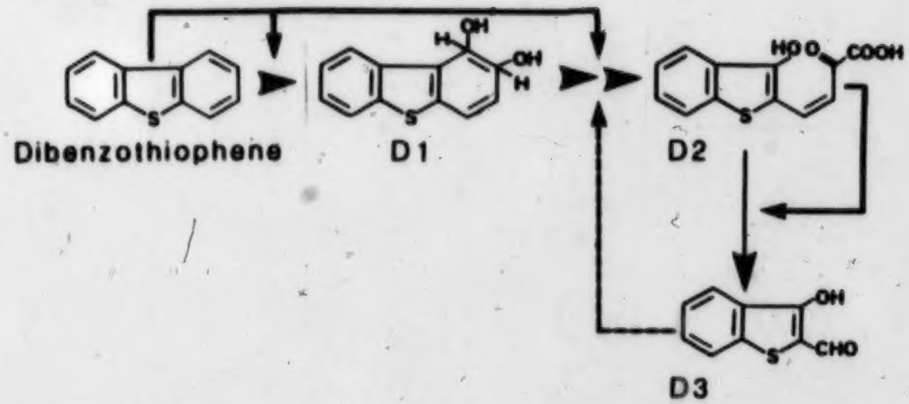

Figure 4.3 Suggested regulation scheme for degradation of dibenzothiophene by *Flavobacterium* (BW-2) phenotypic variant #46. Solid heavy lines indicate positive control of enzyme activity; broken line indicates negative control. Presumptive compound identification: Compound D1: 1,2-dihydroxy-1,2-dihydro-dibenzothiophene; Compound D2: *trans*-4-(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoic acid; Compound D3: 3-hydroxy-2-benzothiophene

constitutive, low levels of the ortho cleavage enzyme produced sufficient amounts of the cleavage product (cis,cis-muconate) to induce further production of the cleavage enzyme ("product induction"; Singer and Finnerty, 1984).

In contrast to variant #46, the wild type produced both intermediates (D1,D2) and the final product (D3), but tended to accumulate the hydroxylated ring structure (D1) without high production of the other two compounds (Figure 4.4). No regulatory scheme was proposed for the wild type.

Variant B accumulated the hydroxylated intermediate (D1) and the ring cleavage product (D2), but showed no evidence for the accumulation of the final product (D3) (Figure 4.4). This variant may be blocked at the regulatory level, or may be a structural mutant.

When the same experiment was performed with 3mM fluorene, the hydrocarbon analogue of dibenzothiophene, there were no striking differences among the three cultures regarding the number of peaks observed (data not presented). The only difference was that variant #46 produced a much larger peak at 470 nm than the wild type or variant B. (Presumably this unidentified peak represents the fluorene ring cleavage compound corresponding to the ring cleavage compound of dibenzothiophene (D2), which has an absorption maximum at 480 nm, and also represents the intense yellow color produced by variant #46 on fluorene sprayplates.) It is surprising that the fluorene scan of variant B was so

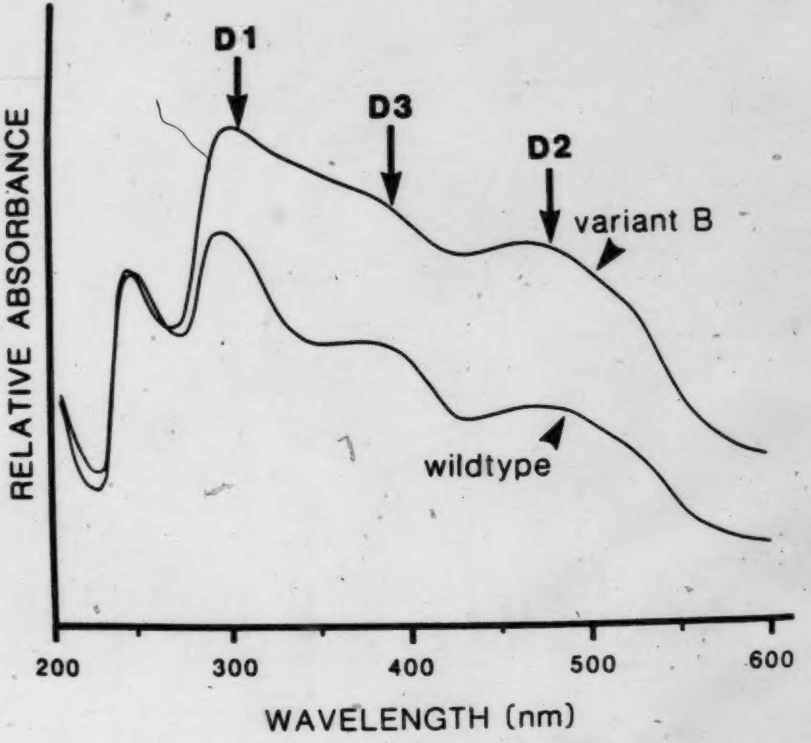

Figure 4.4 Spectrophotometric scan of culture supernatants from *Flavobacterium* (8W-2) wild type and phenotypic variant B incubated with dibenzothiophene. Cultures grown in ASW + 0.01% YE + NP + 2.5mM dibenzothiophene at 26°C in the dark. Samples were removed at intervals, microfuged and blanked versus parallel uninoculated medium. Results from samples removed after 50 hr incubation are illustrated. D1 = 1,2-dihydroxy-1,2-dihydro-dibenzothiophene; D2 = *trans*-4-(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoic acid; D3 = 3-hydroxy-2-formyl- benzothiophene.

similar to that of the wild type, since B produced very little color on fluorene sprayplates, and was unable to clear the fluorene layer, compared with the wild type.

4.3.6.1 Hydroxylation of PAH

The production of hydroxylated aromatic compounds (the first step in oxidative microbial attack on PAH) can be measured with tetraazotized o-dianisidine (t.o.d) (Wackett and Gibson, 1983). This technique was used in combination with spectrophotometric scans to determine the amount of oxidation of several PAH by five cultures of *Flavobacterium* (8W-2) (wild type and variants). This was of particular interest in view of the observation that variant B was able to initiate attack on the dibenzothiophene molecule but was unable to complete the degradation (Table 4.5 and Figure 4.4). The PAH were tested at two concentrations (0.5mM or 5mM) to observe any concentration effects on degradation. Inoculum sizes were determined by plate count immediately before inoculation and all were similar in # viable cells/mL except for variant B-1L, which was approximately 10-fold lower. Figures 4.5, 4.6 and 4.7 present graphs constructed from absorption values at wavelengths specific for degradation intermediates. Unfortunately, there are only three points recorded for the incubation period studied and, therefore, only general trends can be described. Tables 4.7, 4.8, 4.9 and 4.10 present absorption values for t.o.d. derivatized cultures plus the spectrophotometric values from the final incubation period. The molar extinction coefficients of the intermediates and the t.o.d. derivatives are unknown. Therefore, all observations are based on relative absorbances rather than on absolute values.

1. Dibenzothiophene

Table 4.7 presents spectrophotometric results describing the metabolism of dibenzothiophene. In medium containing 0.5mM dibenzothiophene, variant B-1L showed the greatest accumulation of hydroxylated material [1,2-dihydroxy-dibenzothiophene (D1)], as indicated by the t.o.d. reaction, but did not show subsequent high absorbance values at either 480 nm or at 390 nm, the respective absorbance maxima for the ring cleavage product [*trans*-4(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoate (D2)] and final product [3-hydroxy-2-formyl-benzothiophene (D3)].

The graph in Figure 4.5 shows changes in culture medium absorbance at 480 nm with time, which correspond to the level of ring cleavage compound (D2) in the medium. When these results were combined with t.o.d.-derivatized hydroxylated aromatic results in Table 4.7, trends could be discerned regarding the production of dibenzothiophene intermediates. For example, B-1L appeared to be capable of initiating attack on dibenzothiophene by hydroxylation, but showed

Table 4.7 Metabolism of dibenzothiophene by *Flavobacterium* (8W-2) wildtype and four phenotypic variants, measured spectrophotometrically. Cultures incubated in ASW + 0.01% YE + N,P + 0.5mM or 5.0mM dibenzothiophene at 26°C. Hydroxylated products were measured after 69 hr incubation by reaction with tetraazotized o-dianisidine (t.o.d.). Putative ring fission product and final product were measured by absorption at 480 nm and 390 nm, respectively, after 13 d incubation.

	0.5mM Dibenzothiophene			5.0mM Dibenzothiophene		
Inoculum	t.o.d. derivative (69 hr)	fission product (13 d)	final product (13 d)	t.o.d. derivative (69 hr)	fission product (13 d)	final product (13 d)
wt	0.236	0.077	0.280	0.161	0.080	0.240
Maint	0.178	0.157	0.268	N.D.[1]	0.343	0.370
B-1L	0.250	0.035	0.100	0.194	0.025	0.080
T101	0.211	0.043	0.155	0.225	0.146	0.200
T118	0.111	0.037	0.250	0.272	0.020	0.100

[1] N.D. = not determined

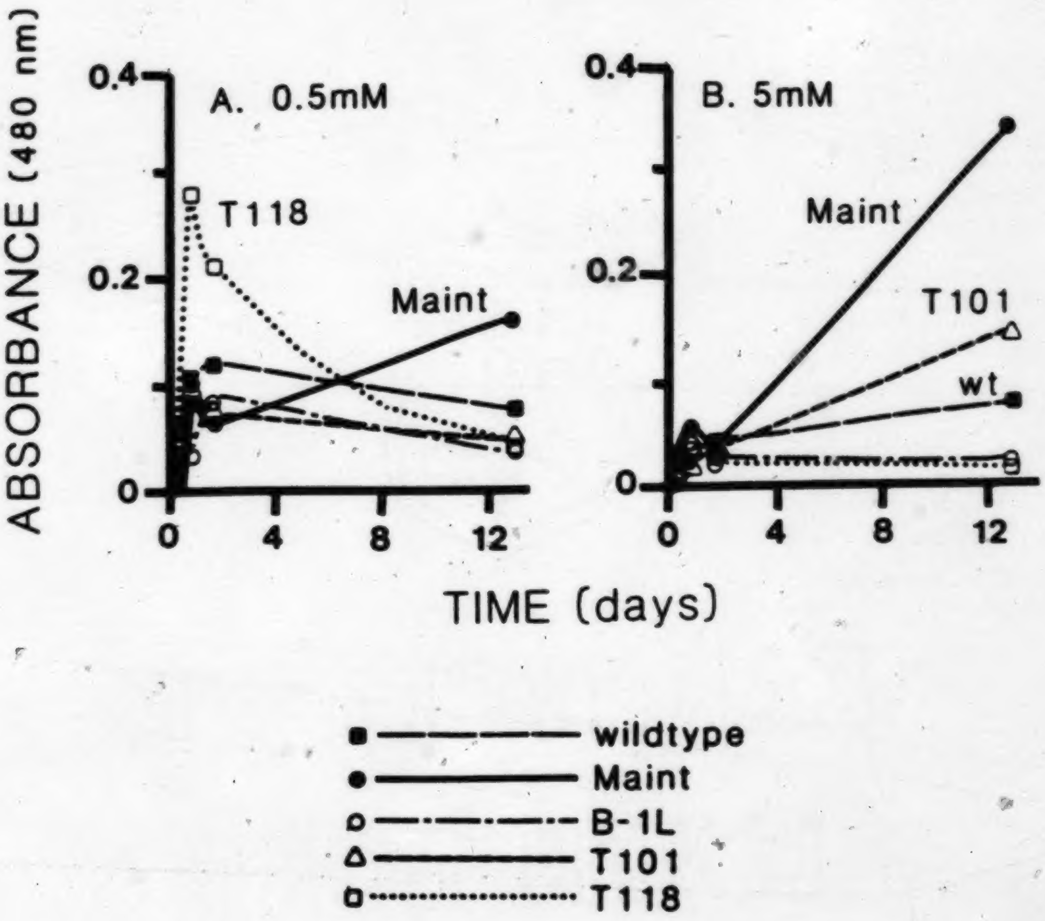

Figure 4.5 Production of dibenzothiophene degradation intermediate by *Flavobacterium* (8W-2) wild type and 4 phenotypic variants. Graph of culture supernatant absorbance versus incubation time for cultures grown in ASW + 0.01% YE + 0.5mM or 5.0mM dibenzothiophene at 26°C. Absorbance at 480 nm represents absorption by the dibenzothiophene ring cleavage product (D2).

only limited metabolism of the hydroxylated product (D1), as suggested by a slow decrease in absorption at 480 nm, presumably as the ring cleavage compound (D2) was metabolized. This compared well with previously discussed results from variant B grown in 2.5mM dibenzothiophene (Figure 4.4). In 5mM dibenzothiophene, the amount of hydroxylated material (D1) decreased, as did both the ring fission product (D2) and the final product (D3). This suggested a slight inhibition of degradation at higher concentrations of dibenzothiophene, as observed by Monticello *et al.* (1985).

Variant T118 grown in 0.5mM dibenzothiophene (Panel A, Figure 4.5) showed an initial surge of fission product which decreased quickly. During the initial surge, the concentration of hydroxylated product (D1) was low presumably due to cleavage, and the final product (D3) was high, indicating quick metabolism of dibenzothiophene with a temporary accumulation of ring fission product (D2). This was paralleled by previous observations with variant #46 (Figure 4.3). However, in medium containing 5mM dibenzothiophene, T118 accumulated the hydroxylated intermediate (D1) and did not produce the ring cleavage (D2) or final product (D3). The accumulation of hydroxylated product (D1) observed in variant #46 was proposed to be the result of feedback control by the final product (D3). In the case of T118, this mechanism is unlikely, since the final product (D3) was present at much lower concentrations at 5mM dibenzothiophene than at 0.5mM dibenzothiophene. Another regulatory mechanism must be involved to stop metabolism after the initial hydroxylation. Again, there was an inhibitory effect on product formation by higher dibenzothiophene concentrations.

Variant T101 showed intermediate levels of all products compared with the other cultures at both concentrations. The fission product (D3) was three times higher in 5mM dibenzothiophene medium than in 0.5mM medium. Therefore, there was not a proportional increase in the product formed. The intermediates increased only marginally in the ten-fold higher concentration of dibenzothiophene. The Maint variant showed a slow accumulation of fission product (D2) with time at both concentrations. The concentration of the final product (D3) was also high in both media. Both products were higher in 5mM media than in 0.5mM media, but not ten-fold higher. This might indicate limited induction of the later degradative genes.

The wild type produced intermediate levels of hydroxylated product (D1). It also produced almost constant levels of fission product (D3) over time, which were approximately equal independent of the substrate concentration. The wild type showed higher levels of final product (D3) at the lower concentration.

Interestingly, the data of Hou and Laskin (1976) also showed a biphasic response to dibenzothiophene concentration. That is, the production of "red conversion products" by *Pseudomonas* ERC 8 showed two straight-line responses to dibenzothiophene concentration, with a break-point at approximately 3mM dibenzothiophene. However, the authors did not discuss this phenomenon, nor did they explain the biphasic response to dibenzothiophene. Kargi and Robinson (1984) reported that a dibenzothiophene-oxidizing *Sulfolobus* isolate was inhibited by initial dibenzothiophene concentrations greater than 2.7mM, and Monticello *et al*. (1985) observed that the products of dibenzothiophene oxidation were inhibitory to the oxidizing cells.

To summarize, all isolates tested in this experiment were capable of the initial hydroxylation of dibenzothiophene. This may represent the activity of constitutive or non-specific dioxygenases, as reviewed by Gibson and Subramanian (1984). Three of five cultures showed decreased levels of final product (D3) at higher initial concentrations of dibenzothiophene, which could indicate feedback inhibition, or substrate (or product) toxicity. In the other two cultures, the increase in final product (D3) was not proportional to the ten-fold increase in dibenzothiophene concentration, but rather represented an increase of only 30-40% over the levels produced at the lower substrate concentration. Isolate B-1L showed a degradative pattern similar to variant B, from which it was derived, while T118 displayed similarities to variant #46. The wild type appeared to hydroxylate and cleave dibenzothiophene at the same rate regardless of the substrate concentration, but produced slightly more final product (D3) at the low concentration. This assortment of phenotypes suggests complex regulatory patterns of induction and feedback inhibition or repression. Either substrate or product, or both, may also have toxic effects on dibenzothiophene metabolism.

2. Phenanthrene

Degradation of phenanthrene is harder to measure spectrophotometrically than dibenzothiophene for several reasons. First, there is no defined end product of phenanthrene degradation to accumulate in the medium, since the substrate theoretically is degraded into small molecular weight compounds which are central to common degradative pathways. Second, intermediates of the phenanthrene degradation pathway [e.g. 1-hydroxy-2-naphthoate (P2), o-phthalate (P3), protocatechuate (P4)] have complex absorption spectra (Table 4.6). Third, the absorption spectra of the early intermediates [eg. the dihydroxy derivatives (P1)] have not been published, and are not available commercially as authentic standards. Nevertheless, an attempt was made to monitor degradation of phenanthrene spectrophotometrically. Two phenanthrene degradation pathways have been reported in the literature. The salicylate pathway was proposed by Evans *et al*. (1965) for *Pseudomonas* spp., and degradation of phenanthrene through o-phthalate was reported for *Aeromonas*, *Alcaligenes*, *Vibrio* and *Micrococcus* spp. (Kiyohara *et al*., 1976, 1978, 1982a; Ghosh and Mishra, 1983). *Flavobacterium* (8W-2) appears to use the latter pathway for phenanthrene metabolism.

Hydroxylation of phenanthrene at two concentrations was measured by the t.o.d. reaction after 69 hr incubation with five cultures of *Flavobacterium* (8W-2) (Table 4.8). All cultures were able to hydroxylate phenanthrene at both concentrations tested. Both the wild type and variant T118 showed a 30% increase in the amount of hydroxylated product (P1) at the higher substrate concentration; the other three cultures showed decreased levels. No other specific trends were noted.

Further degradation of phenanthrene was observed by recording spectrophotometric scans of culture supernatants clarified by centrifugation in a microfuge. A major peak appeared at 330 nm with secondary peaks at 285 and 300 nm, and was identified as 1-hydroxy-2-naphthoate (P2) by comparison with the

Table 4.8 Metabolism of phenanthrene by *Flavobacterium* (8W-2) wild type and 4 phenotypic variants, measured spectrophotometrically. Cultures incubated in ASW + 0.01% YE + NP + 0.5mM or 5mM phenanthrene at 26°C. Hydroxylated products were measured after 69 hr incubation by reaction with tetraazotized o-dianisidine (t.o.d.). Putative ring fission product was measured by absorption at 480 nm after 13 d incubation.

	t.o.d.derivative (69 hr)		absorbance at 480nm (13 d)	
Inoculum	0.5mM Phenanthrene	5mM Phenanthrene	0.5mM Phenanthrene	5mM Phenanthrene
wt	0.183	0.235	0.061	0.045
Maint	0.178	0.158	0.061	0.035
B-1L	0.272	0.188	0.143	0.094
T101	0.233	0.078	0.068	0.052
T118	0.259	0.332	0.086	0.063

authentic standard (discussed below). Longer incubation caused another broad peak to be seen at approximately 480 nm. It was assumed that this peak represented *cis*-4(2-(1-hydroxy)-naphthenyl)-2-oxo-3-butenoate, and was analogous to the ring fission product of dibenzothiophene, *trans*-4(2-(3-hydroxy)-thionaphthenyl-2-oxo-3-butenoate (D2) (which has an absorption maximum at 480 nm) because of the structural and chemical similarity of the two compounds. Relative amounts of this proposed intermediate produced after 13 days incubation are summarized in Table 4.8. Variant B-1L had higher levels of this absorbing compound than the other four cultures, analogous to the accumulation of dibenzothiophene ring fission product noted with variant B. Values for other cultures were low, and decreased at higher substrate concentration.

The appearance of 1-hydroxy-2-naphthoate (P2) is shown in Figure 4.6. The levels of 1-hydroxy-2-naphthoate (P2) produced in 0.5mM phenanthrene (Panel A) were approximately equal for all cultures, although variant B-1L showed a rapid initial accumulation of this intermediate. In 5mM phenanthrene (Panel B), the rate of production of the intermediate varied with the source of the inoculum, with variant B-1L showing the slowest production of 1-hydroxy-2-naphthoate (P2) and Maint showing the fastest rate. There was an approximate two-fold increase in the final 1-hydroxy-

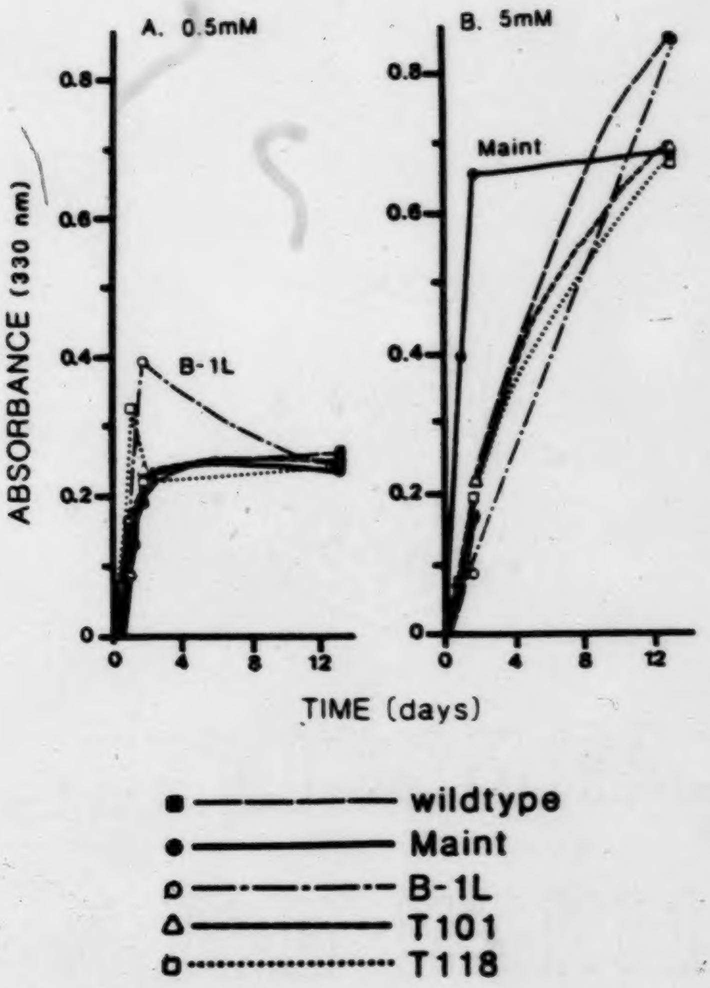

Figure 4.6 Production of phenanthrene degradation intermediate by *Flavobacterium* (8W-2) wild type and 4 phenotypic variants. Graph of culture supernatant absorbance versus incubation time for cultures grown in ASW + 0.01% YE + 0.5mM or 5.0mM phenanthrene at 26°C. Absorbance at 330 nm represents absorption by 1-hydroxy-2-naphthoate.

2-naphthoate (P2) levels with a ten-fold increase in substrate concentration. The apparent levelling off of 1-hydroxy-2-naphthoate (P2) levels in the 0.5mM phenanthrene cultures could be explained by different effects: (a) a shutdown in production of the intermediate and its degradation, so that levels were unaltered; or (b) depletion of the intermediate levels at a rate equal to its production, to keep levels constant. This experiment could not distinguish between the two possibilities. However, spectrophotometric scans of 13 day old cultures incubated with 0.5mM phenanthrene (data not presented) showed additional peaks at approximately 286 and 252 nm, corresponding to the absorption spectrum of protocatechuate (P4), another degradative intermediate. The only exception was variant B-1L, which only showed peaks corresponding to 1-hydroxy-2-naphthoate (P2). This suggested that the first explanation was most likely to fit variant B-1L, and for the remaining four cultures the second explanation for constant 1-hydroxy-2-naphthoate levels was more likely. Spectrophotometric scans of 13 day old cultures incubated with 5mM phenanthrene did not show profiles resembling protocatechuate (P4), possibly explaining the accumulation of 1-hydroxy-2-naphthoate (P2) in these cultures. That is, degradation of the 1-hydroxy-2-naphthoate (P2) was reduced or stopped at high phenanthrene concentrations. Many similarities were observed between dibenzothiophene and phenanthrene degradation by these five cultures.

3. Naphthalene

Hydroxylation of naphthalene as measured by reaction with t.o.d. is presented in Table 4.9. Values were similar for all cultures at both substrate concentrations, with the exception of a high value for variant B-1L at 5mM naphthalene. Selected cultures were observed after 13 days incubation by measuring absorption at 294 nm (an absorption maximum for salicylate, a degradation intermediate). The Maint variant showed equal absorbances at both naphthalene concentrations, while variant T118 showed high absorbance at 0.5mM but 3-fold lower absorbance in 5mM naphthalene. Variant B-1L showed the highest absorption in 5mM naphthalene; it is likely that B-1L was unable to metabolize salicylate, since it appeared to accumulate in the medium.

Absorbance of the culture medium was recorded at 370 nm where an absorption plateau was observed. Unfortunately, when this wavelength was subsequently compared with absorption maxima of authentic pathway intermediates, it did not correspond to any particular compound. Therefore, the graph of culture medium absorbance versus time is not presented, although the trends did parallel the results detailed in Table 4.9.

Table 4.9 Metabolism of naphthalene by *Flavobacterium* (8W-2) wild type and 4 phenotypic variants, measured spectrophotometrically. Cultures incubated in ASW + 0.01% YE + NP + 0.5mM or 5mM naphthalene at 26°C. Hydroxylated products were measured after 69 hr incubation by reaction with tetraazotized o-dianisidine (t.o.d.). Salicylate was measured by absorption at 294 nm after 13 d incubation.

	t.o.d. derivative (69 hr)		absorbance at 294nm (13 d)	
Inoculum	0.5mM Naphthalene	5mM Naphthalene	0.5mM Naphthalene	5mM Naphthalene
wt	0.135	0.154	ND[1]	ND
Maint.	0.163	0.127	0.146	0.130
B-1L	0.193	0.230	ND	0.55
T101	0.169	0.127	ND	ND
T118	0.168	0.160	0.30	0.09

[1] ND = not determined

No regulatory schemes for the degradation pathway are presented, but it appears from t.o.d. results that all cultures were capable of hydroxylating naphthalene at similar rates. It is assumed that *Flavobacterium* (8W-2) uses the salicylate pathway for degradation of naphthalene.

4. Biphenyl

The t.o.d.-reacted values for cultures incubated with biphenyl are presented in Table 4.10. At either substrate concentration, variant B-1L showed the highest levels of hydroxylated biphenyl. The other four cultures had lower, equivalent values. All values except for the wild type decreased at higher substrate concentrations. This could indicate either induction of the enzymes for degradation of the hydroxylated intermediate, or inhibition of the hydroxylating enzymes in 5mM biphenyl. The difference might have been observed by measuring mineralization of labelled biphenyl at different substrate concentrations.

Cultures were observed spectrophotometrically at 420 nm, the absorption maximum for the biphenyl ring cleavage products, (α-hydroxy-β-phenylmuconic acid and 2-hydroxy-6-phenyl-hexa-2,4-dienoate; spectrophotometric analysis cannot discriminate between these two fission products). Results are shown in Figure 4.7. In 0.5mM biphenyl medium (Panel A), after an initial accumulation of cleavage products (especially with

Table 4.10 Metabolism of biphenyl by *Flavobacterium* (8W-2) wild type and 4 phenotypic variants, measured spectrophotometrically. Cultures incubated in ASW + 0.01% YE + NP + 0.5mM or 5mM biphenyl at 26°C. Hydroxylated products were measured after 69 hr incubation by reaction with tetraazotized o-dianisidine (t.o.d.).

	Concentration of Biphenyl	
Inoculum	0.5mM	5.0mM
wild type	0.161	0.162
Maint	0.205	0.157
B-1L	0.322	0.280
T101	0.217	0.176
T118	0.232	0.190

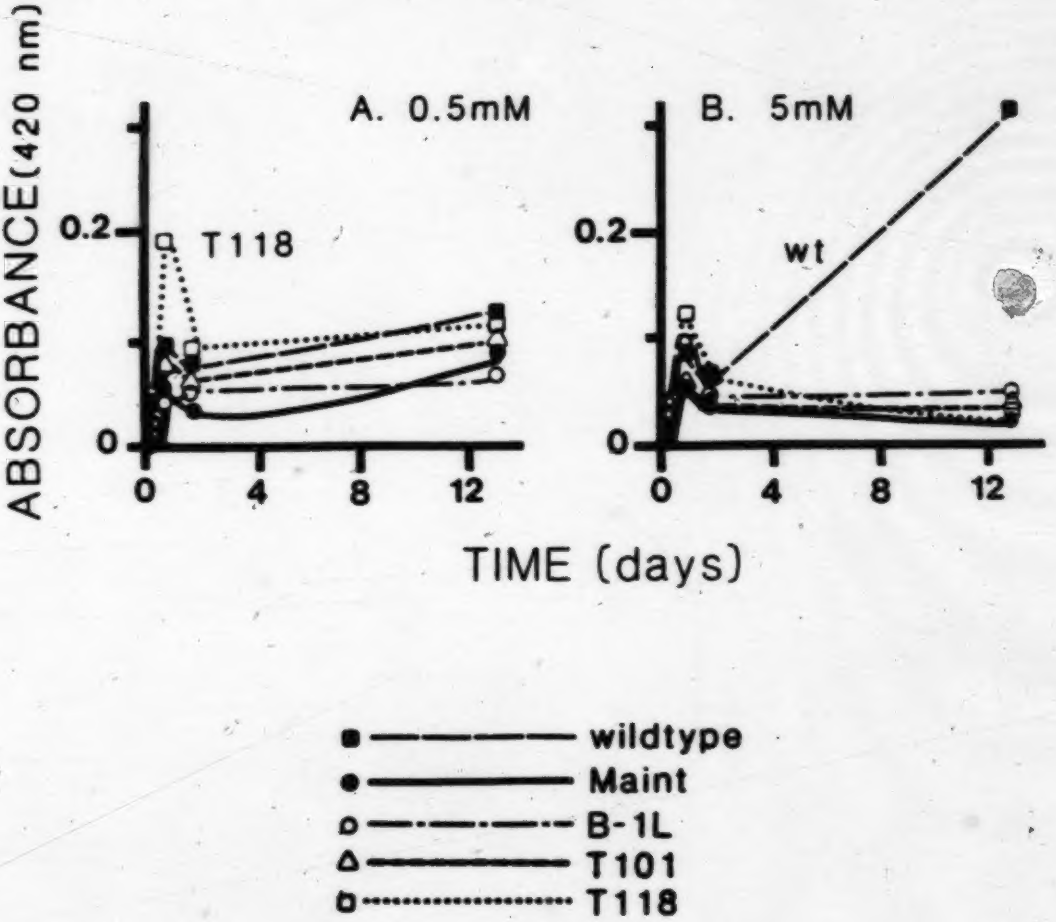

Figure 4.7 Production of biphenyl degradation intermediate by *Flavobacterium* (8W-2) wild type and 4 phenotypic variants. Graph of culture supernatant absorbance versus incubation time for cultures grown in ASW + 0.01% YE + 0.5mM or 5.0mM biphenyl at 26°C. Absorbance at 420 nm represents absorption by the biphenyl ring cleavage product(s).

T118), the amount of intermediate decreased and levelled off. The same pattern was seen at approximately the same levels in 5mM biphenyl medium (Panel B), except that the wild type appeared to accumulate the ring cleavage product. Spectrophotometric scans of the 13 day cultures showed no distinctive absorption maxima, but, rather, gently sloping composite curves. There was no evidence for accumulation of other pathway intermediates such as benzoate or catechol, suggesting that the biphenyl was completely degraded, presumably via catechol.

The similarity of t.o.d. values, and results from absorbance at 420 nm suggest that regulation of biphenyl degradation is similar to that of naphthalene. That is, all *Flavobacterium* (8W-2) cultures initiate degradation by hydroxylation with constitutive or non-specific oxygenases which may not be induced by growth on biphenyl, then completely degrade the biphenyl. It is possible that induction of the pathway is achieved by a pathway intermediate, for example the ring cleavage product which initialy accumulates, then decreases through metabolism. Alternatively, another pathway intermediate such as benzoate or catechol could induce metabolism of the ring cleavage product.

Summary of PAH hydroxylation studies

Some general conclusions were drawn regarding PAH metabolism by these cultures, and variant B-1L in particular. This strain tended to accumulate the hydroxylated intermediates in the first stages of PAH attack. With biphenyl, it was able to slowly degrade the accumulated hydroxylated compound without apparent accumulation of other intermediates, whereas with dibenzothiophene, phenanthrene and naphthalene, it was able to degrade the initial oxidative product slowly, but then accumulated later pathway intermediates, suggesting distal enzymic blocks. No conclusions were reached with data from these experiments regarding the nature of these blocks, whether regulatory or structural. Variant T118 tended to accumulate the hydroxylated intermediates only at high substrate concentrations of dibenzothiophene and phenanthrene, which may be a reflection of regulatory mechanisms or of product or substrate toxicity. It certainly is possible that, since all variants were capable of performing the initial hydroxylations of four different PAH, the hydroxylating enzymes are non-specific dioxygenases or that the genes are constitutive and chromosomal. [Gibson and Subramanian (1984) in reviewing enzymatic oxidation of aromatic hydrocarbons concluded that several of the multicomponent dioxygenases and dehydrogenases responsible for hydroxylative attack on aromatic compounds have a wide inducer and substrate specificity.] Pathway regulation would appear to be complex and further complicated by evidence of substrate or product toxicity at higher concentrations.

4.3.6.2 PAH concentration effects

To examine the effects of increasing substrate concentration on production of pathway intermediates, variant T118 was incubated with four concentrations of phenanthrene (0.25mM, 0.5mM, 2.5mM or 5.0mM) in ASW + NP + 0.01% YE at 26°C. Culture aliquots periodically were clarified in a microfuge and supernatants subjected to spectrophotometric scans from 600 to 275 nm, measuring the absorbance of peaks at 480 nm (for the presumed ring cleavage compound) and at 330 nm (for the next intermediate, 1-OH-2-naphthoate).

Results of these scans are shown in Figure 4.8. Panel A shows the accumulation of the presumed ring cleavage product, while Panel B shows accumulation of 1-OH-2-naphthoate (P2). In 0.25mM and 0.5mM cultures, the low levels of (presumed) ring cleavage product remain virtually constant (Panel A). This was paralleled by a rapid initial production of 1-OH-2-naphthoate (P2), followed by depletion, then relatively constant levels of 1-OH-2-naphthoate (Panel B). The pattern of 1-OH-2-naphthoate (P2) production in 0.5mM medium agreed closely with results from a previous experiment in the same medium, in both quantity and temporal appearance. These results imply that an initial accumulation of 1-OH-2-naphthoate was

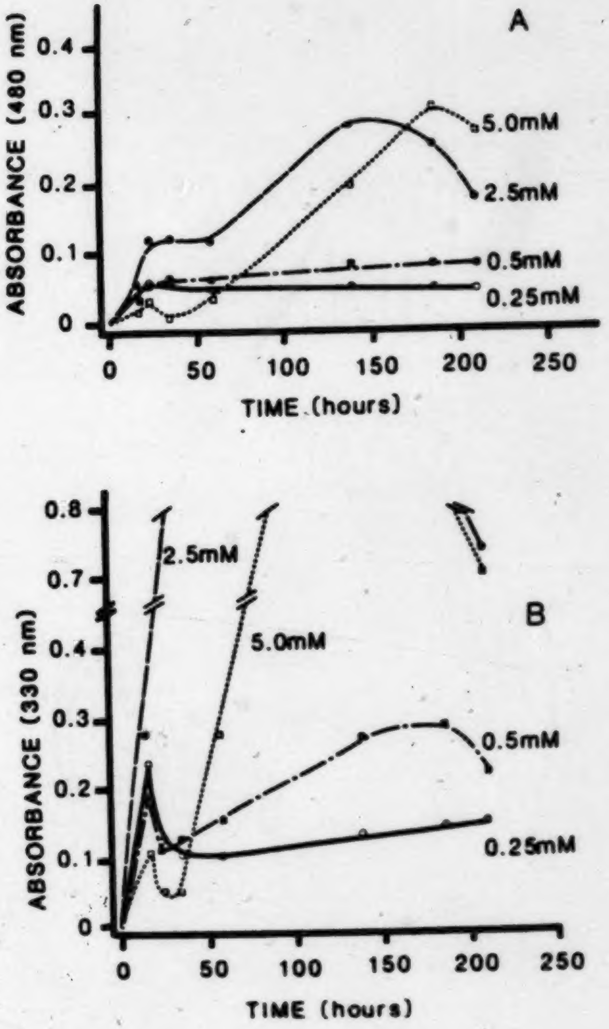

Figure 4.8 Metabolism of phenanthrene at 4 concentrations by *Flavobacterium* (8W-2) variant T118, measured spectrophotometrically. Culture medium consisted of ASW + 0.01% YE + NP + 0.25mM, 0.5mM, 2.5mM or 5.0mM phenanthrene. Absorbance at 480nm (Panel A) presumably represents absorption by the ring cleavage product, and at 330nm (Panel B) by 1-hydroxy-2-naphthoate. Values measured between 100 and 200 hr incubation (Panel B) were ≥ 2.0 and are not shown.

followed by induction of the genes for degradation of the product at a rate sufficient to yield constant levels of intermediates.

A different pattern was observed in 2.5mM and 5.0mM cultures where high levels of the ring cleavage product (P3; Panel A) and 1-OH-2-naphthoate (P2; Panel B) accumulated before depletion was observed. These accumulations and subsequent depletions appeared to be temporally coordinated. This might be explained if metabolism of the 1-OH-2-naphthoate (P2) is the rate-limiting step, and that its precursors, the ring cleavage product and hydroxylated phenanthrene (P1) accumulate (as previously determined), unaffected by any feedback regulation. The rate-limiting step at degradation of 1-OH-2-naphthoate (P2) could be explained by either of two mechanisms.

1. The enzyme is constitutively produced at low levels in T118, and is not induced by growth on phenanthrene.
2. The enzyme is not induced by precursors to 1-OH-2-naphthoate (P2), but is induced by one of its metabolites. In this case, constitutive enzyme levels would have to build up sufficient levels of the inducer before stimulation of 1-OH-2-naphthoate degradation was observed. Release of the rate-limiting step would presumably then allow depletion of the accumulated intermediates.

A regulatory system like this has been demonstrated with *ortho* cleavage of catechol in *Pseudomonas* spp. (Chapter 1), and is the basis for benzoate curing in *P putida*. It has also been reported as salicylate induction of naphthalene metabolism in *Pseudomonas* spp. (Barnsley, 1975). This regulatory scheme would resemble that shown in Figure 4.3, except that no feedback inhibition would be observed, since no defined endproduct of phenanthrene is normally produced. It is not known whether this scheme, proposed for variant T118, represents a possibility for the wild type, and whether it is specific for phenanthrene.

4.3.6.3 Growth on PAH degradation pathway intermediates

The degradation of pathway intermediates by variants was studied in tubes containing ASW ± 0.01% YE plus 1mM or 5mM substrate. The substrates included 1-OH-2-naphthoate, o-phthalate and protocatechuate (phenanthrene pathway intermediates P2, P3 and P4), 1,2-di-OH-naphthalene, salicylate and catechol (naphthalene pathway intermediates), benzoate (an intermediate of biphenyl degradation), and 2,3-di-OH-naphthalene (from the anthracene degradation pathway). Intermediates from the dibenzothiophene pathway were not available commercially. The tubes were inoculated with ASW suspensions of *Flavobacterium* (8W-2) variants, and initially were screened for growth and color production on these intermediates. Some tubes were subsequently monitored for appearance of metabolites by spectrophotometric scans. Selected results are summarized in Table 4.11, which shows that the phenotypes of the variants are mixed. The results are discussed below and considered in conjunction with results from preceeding experiments in terms of pathway regulation and growth of specific variants.

1. Phenanthrene pathway intermediates

The *Flavobacterium* (8W-2) wild-type was unable to grow in 5mM 1-OH-2-naphthoate (P2), but grew in 1mM medium. Similarly, Maint and variant pv43 were unable to grow in 5mM protocatechuate (P4), yet grew in 1mM substrate. This observation may provide further evidence for the postulated inhibitory effect of high phenanthrene concentrations: two of the pathway intermediates appear to be inhibitory at high concentrations. All variants tested were able to grow on o-phthalate (P3); the degradative enzyme may be chromosomally encoded. All variants in Table 4.11 were capable of growth in 1mM protocatechuate (P4) but 12 of 23 other variants also tested were unable to grow on 5mM protcatechuate (and were not retested at 1mM). It is not known whether this is a case of uninduced degradation, loss of the structural gene, or chemical toxicity.

Incubation with 1-OH-2-naphthoate (P2) revealed unexpected phenotypes. For example, variant Maint was

Table 4.11 Growth of *Flavobacterium* (8W-2) wild type and eight phenotypic variants with PAH degradative pathway intermediates as sole carbon source. Cultures grown in ASW + 0.01% YE plus 1mM or 5mM substrate, and incubated at 26°C for 1 week.

	PHENANTHRENE PATHWAY			NAPHTHALENE PATHWAY				ANTHRACENE PATHWAY
						BIPHENYL PATHWAY		
Inoculum	1-OH-2-Naphthoate	o-Phthalate	Protocatechuate	1,2-di-OH-Naphthalene	Salicylate	Catechol	Benzoate	2,3-di-OH-Naphthalene
wild type	-[1,2]	+	+	±	+	ND	-	ND
Maint	±	+	-[2]	-	+	-	-	-
#46	+	+	+	-	±	±	-	+
B-1L	-	+	+	-	-	-	-	-
T118	±	+	+	-	±	ND	ND	ND
pv40	+	+	+	-	+	-	-	-
pv43	-	+	-[2]	±	-	-	-	-
pv64	±	+	+	-	±	-	-	-
pv80	-	+	+	-	+	-	-	-

[1] - = no growth; ± = poor growth; + = growth; ND = not determined

[2] no growth observed at 5mM; growth observed at 1mM

capable of mineralizing phenanthrene (Figure 2.6) and therefore must posess enzymes for degrading 1-OH-2-naphthoate (P2), yet was capable of only minimum growth on the intermediate alone. Similarly, variants pv43, pv64 and pv80 were capable of growing on phenanthrene-sprayed minimal salts agar (Table 4.5) and therefore must be able to degrade 1-OH-2-naphthoate, but showed little or no growth on the intermediate alone. This implies that phenanthrene-grown cultures are induced for 1-OH-2-naphthoate degradation, but that in 7 of 9 cultures, 1-OH-2-naphthoate does not induce its own degradative enzymes. Regarding the two exceptions, variant #46 has been proposed as a regulatory mutant (Figure 4.3) and pv40 may also be a regulatory mutant; both may be constitutively induced for 1-OH-2-naphthoate degradation. Variant B-1L was incapable of significant phenanthrene degradation (Figure 4.1), and T118 produced low levels of $^{14}CO_2$ from labelled phenanthrene (Section 4.3.6). Since both variants were capable of hydroxylating and clearing phenanthrene (Table 4.8), and of growing on o-phthalate (P3) and protocatechuate (P4) (Table 4.11), their limited utilization of 1-OH-2-naphthoate (P2) is a plausible explanation for low levels of phenanthrene mineralization. The variants must have constitutive, low levels of enzymes to degrade 1-OH-2-naphthoate, as suggested by the fact that mineralization of phenanthrene does occur. Perhaps these variants represent regulatory mutants, such that degradation of 1-OH-2-naphthoate is not induced by growth on phenanthrene, or by metabolites. Alternatively, the variants may have leaky point mutations, allowing induced production of low levels of degradative enzymes.

2. Naphthalene pathway intermediates

Barnsley (1983) states that naphthalene and phenanthrene degradative pathways are separately controlled and induced in *Pseudomonas* spp. and certain unclassified Gram negative organisms. Therefore there is no reason to expect a Phn$^-$ variant to have a corresponding Nah$^-$ phenotype.

Only the wild type and variant pv43 were capable of limited growth on 1,2-di-OH-naphthalene, yet all cultures (except B-1L) are known to degrade naphthalene (Table 4.5 and Table 4.9) without accumulation of 1,2-di-OH-naphthalene. Therefore, the variants must posess the genes for degradation of the 1,2-di-OH-naphthalene, but these are not strongly induced by growth on the substrate. Variant B-1L could not grow on naphthalene, and accumulated 1,2-di-OH-naphthalene and salicylate. It may represent a regulatory or leaky structural mutant such as those proposed for Phn$^-$ mutants. Cultures incubated with catechol did not induce the metabolism of catechol, yet these cultures must be capable of degrading catechol when grown on naphthalene or salicylate, since catechol does not accumulate.

Of 28 variants tested, all but B-1L and pv43 were capable of growing on salicylate alone, although some variants grew very slowly or produced colors different from those of the wild type. When these cultures were analyzed spectrophotometrically, different pathway intermediates were observed. Specifically, variant pv64 grew slowly on salicylate and accumulated the next intermediate, catechol. T118 also grew slowly on salicylate and accumulated an intermediate which was not identified (but was neither catechol nor gentisate, an alternate metabolite of salicylate). Variant pv40 produced a color different from the wild type, but no intermediate was identified. Since none but pv64 accumulated catechol when grown in salicylate, it seems reasonable to conclude that salicylate normally induces degradation of catechol, its metabolite. In pv64, either the regulatory or structural genes may be faulty. The fact that B-1L accumulated salicylate when grown on naphthalene (Table 4.9) and could not degrade salicylate alone (Table 4.11) implies that it is deficient in genes for degradation of salicylate. It is suggested that pv43 also lacks the genes for degradation of salicylate. Salicylate may play a central role in the regulation of naphthalene degradation by *Flavobacterium* (8W-2), as suggested by Barnsley (1975) for *Pseudomonas* spp.

3. Biphenyl and anthracene pathways

As discussed previously regarding curing efforts in benzoate medium, the wild type of *Flavobacterium* (8W-2) was not able to grow with benzoate (a biphenyl degradation product) as sole carbon source, and apparently neither could its variants. This may represent lack of induction of benzoate metabolism in benzoate-incubated cells, since benzoate was not seen to accumulate in biphenyl-grown cultures. No other intermediates of biphenyl metabolism were tested, and therefore no conclusions can be drawn regarding regulation.

Only variant #46 was capable of growing slowly on 2,3-di-OH-naphthalene, a metabolite of anthracene. This may represent an escape from enzyme specificity, allowing variant #46 to utilize 2,3-dihydroxy-naphthalene via a dioxygenase normally specific for 1,2-dihydroxy-naphthalene (a metabolite of naphthalene) or 1,2-dihydroxy-phenanthrene, whereas the wild type is unable to cleave this ring structure (Chapter 2). However, without further study, no conclusions can be made regarding the regulation of anthracene degradation by *Flavobacterium* (8W-2).

4.3.7 Correlation of Phenotype with Plasmid Content

Many of the phenotypic variants discussed above were screened for plasmid content by the modified Casse method. Plasmid profiles of variants were compared with wild type profiles in an attempt to correlate phenotypes with plasmid content. In theory, this would allow assignation of phenotypic functions to specific plasmids.

The plasmids of the wild type *Acinetobacter* (8W-1) and three phenotypic variants are shown in Plate 4.2. Comparison of the wild type with other phenotypic variants (data not presented) revealed changes in the plasmid profiles such as loss or gain of plasmids, or shift in apparent molecular weight. For example, variant #9 lost plasmid p8W1-3; variants #40 and #12 showed a complete plasmid profile compared with the wild type, but the relative brightness of the plasmid bands was different. However, none of the changes could be absolutely correlated with observed phenotypic changes in biphenyl or 4-methylbiphenyl metabolism (Table 4.2). More information was needed about the phenotypes of the variants in order to assign functions to the plasmids. Because of this shortcoming, the plasmids of *Acinetobacter* (8W-1) remained cryptic, although circumstantial evidence implicated plasmids p8W1-1 and p8W1-3 in degradative functions.

Similarly, insufficient data were generated to assign a function to the single large plasmid p38W-1 of *Aeromonas* (38W-HC10-1). Difficulties encountered in recognizing

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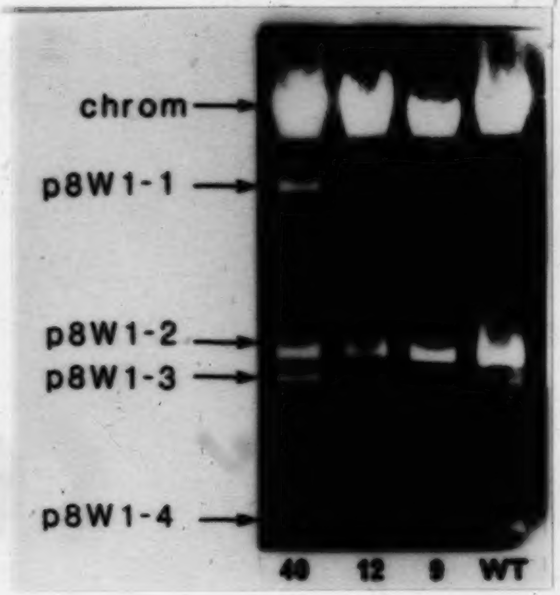
chrom
p8W1-1
p8W1-2
p8W1-3
p8W1-4
40
12
9
WT

Plate 4.2 Plasmids of *Acinetobacter* (8W-1) wild type and phenotypic variants #40, #12 and #9. Photograph of 0.7% agarose gel electrophoresed in TEAS buffer.

phenotypic variants of this isolate (due to its limited PAH degradative capability) caused problems in defining its phenotype. The plasmids of the isolated phenotypic variants were indistinguishable from the wild type plasmid in size (as determined by relative mobility of the intact plasmid in agarose; data not presented) and in restriction digest patterns (Chapter 5). None of the variants showed loss of the plasmid, change in size, or acquisition of a new plasmid. Therefore, the plasmid of *Aeromonas* (38W-HC10-1) also remained cryptic, since there was not enough evidence that PAH degradation in *Aeromonas* (38W-HC10-1) is either plasmid- or chromosome-encoded, or is a result of a combination of these loci.

Although a wide variety of plasmid profiles was observed with *Flavobacterium* (8W-2) phenotypic variants as shown in Plate 4.3, five particular plasmid sizes were observed frequently, occurring in different combinations. Plasmids demonstrating the same mobilities were assumed to be the same species, and plasmids different from the wild type plasmids (pF3, pF4, and pF5) were assumed to be derived from the wild type plasmids by deletions, duplications or insertions (e.g. of chromosomal material). Some "new" plasmids also may represent the excision products of chromosomally integrated plasmids. Such rearrangements of degradative plasmids have been reported previously (Jeenes and Williams, 1982). In particular, the TOL plasmid is capable of undergoing deletions and duplications (mediated

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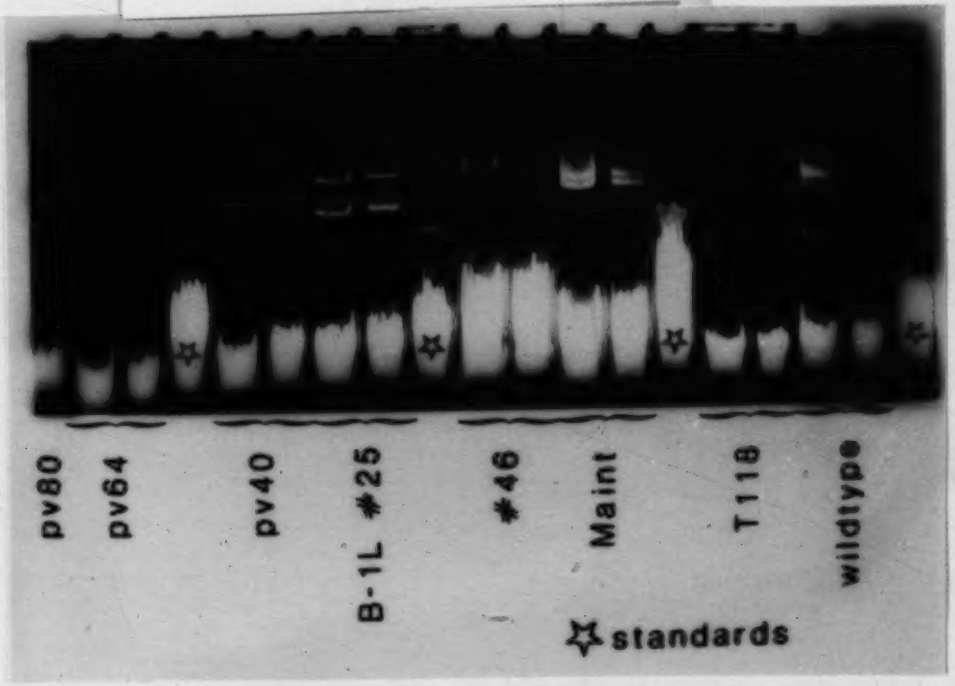
pv80
pv64
pv40
B-1L #25
#46
Maint
T118
wildtype
☆ standards

Plate 4.3 Plasmids of *Flavobacterium* (8W-2) wild type and 7 phenotypic variants. Photograph of 0.7% agarose gel electrophoresed in TEB buffer. Paired lanes contain different volumes of plasmid preparation. Lanes marked ☆ contain molecular weight marker plasmids (166Mdal, 112Mdal, 78Mdal and 33Mdal).

by inverted repeats), leading to heterogeneous plasmid populations (Lehrbach *et al.*, 1982). Because of the complexity of the *Flavobacterium* (8W-2) plasmid profiles and the apparent complexity of the degradative phenotypes regarding regulation and degree of expression, no plasmid species could be correlated positively with a specific phenotype.

Subclones of single phenotypic variants (discussed in section 4.3.5) which demonstrated different phenotypes also displayed subtle changes in plasmid profiles (Plate 4.4), such as change in apparent molecular weight (variant T118, lanes 1 and 2) or in number of plasmid species (variant B-1L, lanes 3, 4, 5 and 6). Even within these subclones, phenotypic variations could not be ascribed to a particular plasmid change.

Other researchers have encountered difficulty in localizing degradative genes in plasmid-containing bacteria. Furukawa *et al.* (1983) have reported that biphenyl, xylene/toluene and salicylate metabolism is regulated by a common unit in *Pseudomonas paucimobilis* strain Q1 [which, incidentally, is morphologically very similar to *Flavobacterium* (8W-2), exhibiting the same cell envelope pigment absorption maxima, growth temperature optimum and mercury resistance characteristics, and many similar biochemical activities]. Strain Q1 contains 3 plasmids (100 Mdal, 45 Mdal and 20 Mdal) which do not exhibit homology with TOL. Although Furukawa *et al.* (1983) could demonstrate common

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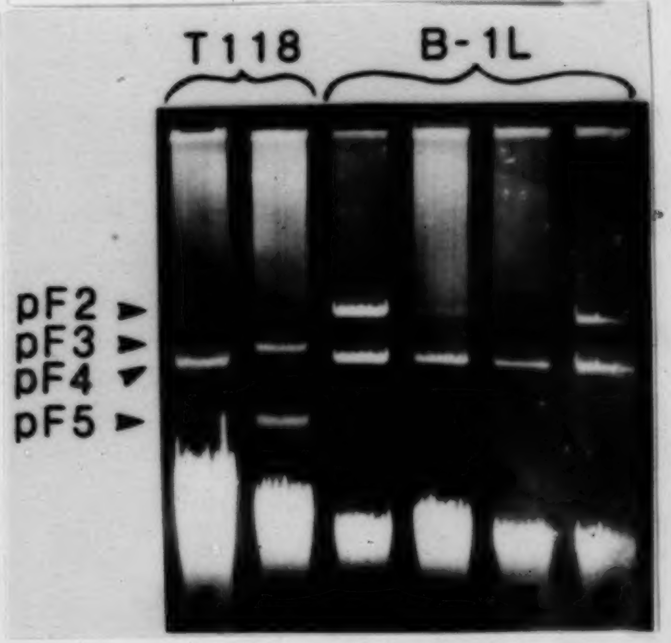

Plate 4.4 Plasmids of *Flavobacterium* (8W-2) subclones from phenotypic variants T118 and B-1L. Photograph of 0.7% agarose gel electrophoresed in TEB buffer. pF-2 90Mdal; pF-3 74Mdal; pF-4 55Mdal; pF-5 30Mdal.

induction and regulation of aromatic degradation pathways in this strain, they were unable to prove that any of the 3 plasmids actually encodes the degradative genes. They proposed that phenotypic variants of strain Q1 produced by SDS or mitomycin-c treatment were the result of loss of entire degradative gene segments or of insertion/deletion mutations in regulatory loci, but had no proof of this hypothesis. Kiyohara *et al.* (1981) could not prove involvement of plasmids in Phn$^+$ *Alcaligenes faecalis* AFK2, or in other genera (Kiyohara *et al.*, 1983). Ghosh and Mishra could not prove plasmid involvement in phenanthrene oxidation by a *Micrococcus* sp.

It is unfortunate the the complexity of the results precluded correlation of plasmid content with PAH degradative function in *Flavobacterium* (8W-2). More accurate definition of phenotypes undoubtedly would have provided better information on which to base deductions regarding degradative functions. Using data from this study, it is not clear to what extent the PAH degradative phenotype in *Flavobacterium* (8W-2) is plasmid encoded, nor which of the plasmids is (are) involved. It is possible that the degradative functions are distributed among the plasmids of *Flavobacterium* (8W-2) rather than being limited to a single plasmid species. It is also possible that duplications of genes (on the chromosome or on disparate plasmid species or on both) account for the lack of correlation between plasmid profile and phenotype. Chromosomal involvement with degradative plasmids has been demonstrated with alkane degradation by OCT, where "highly constitutive levels of alcohol-oxidizing activity in OCT$^-$ strains were attributed to redundant chromosomal... genes" (Singer and Finnerty, 1984). Degradation of phenol also has been shown to result from the complex regulation of 2 or more isofunctional enzymes mediating several steps of the pathway (Hughes *et al.*, 1984); in some bacteria these isofunctional enzymes are chromosomal (Wong and Dunn, 1976).

It is unlikely that all PAH degradative functions in *Flavobacterium* (8W-2) are chromosomal because of the frequency of appearance of phenotypic variants, but very likely that the chromosome is involved either through regulatory functions or encodes structural genes for degradative enzymes. This probable chromosomal involvement (for example, with o-phthalate and salicylate metabolism, and PAH hydroxylative enzymes; discussed in section 4.3.6 and Table 4.11) also complicates the assignation of degradative functions.

The postulated cooperation of degradative loci might explain the observed stability of the degradative phenotype in the absence of selection (e.g. during growth in rich, complex medium without PAH; Chapter 2) because the loss of a plasmid would not eliminate all degradative capability. Cooperativity might also account for the diverse PAH degradative capabilities of the *Flavobacterium* (8W-2) isolate, since combinations of functions are theoretically possible, allowing adaptation to a variety of aromatic substrates. Alternatively, the ability to degrade a variety of substrates may be due to the production of broad-specificity enzymes (Williams and Murray, 1974) such as hydroxylating enzymes, or to production of isofunctional enzymes (Poh and Bayly, 1980; Hughes *et al*., 1984) or both, as previously suggested.

The involvement of mobile genetic elements (i.e. transposons) encoding PAH degradative genes in *Flavobacterium* (8W-2) has not been postulated yet, since the situation is already sufficiently complex. However, there is precedent for speculation about the existence of transposons in degradative plasmids, because TOL contains direct repeats which mediate spontaneous, precise insertions and deletions (Lehrbach *et al*., 1982). Such transposition elements may exist in *Flavobacterium* (8W-2), possibly accounting for such phenomena as the changing plasmid profiles and instability of the mercury resistance phenotype. Chapter 5 presents further speculation about the possible role of transposons in *Flavobacterium* (8W-2). Certainly, studies dealing with homology between the *Flavobacterium* (8W-2) chromosome and the different plasmid species might have provided information regarding duplications or interrelationships of degradative genes in different loci, and on the existence of mobile elements in *Flavobacterium* (8W-2). However, these types of experiments were precluded by the technical difficulties discussed in Chapter 5, regarding the

Flavobacterium plasmids. Alternatively, transposon mutagenesis could have been used as a specific probe for promoting phenotypic variation, and identifying the corresponding genotypic location. Both of these studies would be recommended strongly for further examination of the role of plasmids in PAH degradation by *Flavobacterium* (8W-2) especially, or by other isolates.

4.3.8 Summary

A variety of curing techniques were used to generate phenotypic variants of *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1). No regulatory scheme was proposed for biphenyl degradation by *Acinetobacter* (8W-1) and degradative functions could not be ascribed to particular plasmid(s).

Aeromonas (38W-HC10-1) was found to posess degradative enzymes for the lower steps of PAH pathways, but the location of the genes could not be determined, although genes for growth on benzoate and o-phthalate may be chromosomal.

Variants of *Flavobacterium* (8W-2) demonstrated inconsistent antibiotic- and metal-resistance phenotypes, which were apparently unrelated to plasmid content, and may be either chromosomally encoded or mobile (e.g. transposons). Some phenotypic variants of *Flavobacterium* (8W-2) demonstrated a physical change in cell surface morphology, implying that some PAH$^-$ phenotypes may be the result of "secondary" changes in cell permeability or transport mechanisms rather than degradative enzyme alterations.

The observation that naphthalene and phenanthrene served as sole carbon source for all *Flavobacterium* (8W-2) variants tested implies that at least some PAH degradative enzymes are chromosomal or are duplicated. Different levels of expression of the PAH phenotypes suggested sophisticated regulatory control in *Flavobacterium* (8W-2), possibly consisting of induction and feedback regulation. However, observation of the initial hydroxylative attack on several PAH by all *Flavobacterium* (8W-2) variants tested probably indicates the presence of a constitutive low level dioxygenase (of undetermined specificity) which may be chromosomal.

Degradation of several pathway intermediates apparently was not induced by growth on those intermediates (e.g. 1-hydroxy-naphthoate, 1,2-dihydroxy-naphthalene, catechol and possibly benzoate); this has further implications for degradative regulation.

Unfortunately, correlation of specific plasmids with PAH degradative phenotypes was not possible with *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) or *Aeromonas* (38W-HC10-1). The use of homology studies and transposon mutagenesis is recommended for future studies.

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Chapter 5

Plasmid Characterization Studies

5.1 Introduction

Plasmids may be characterized with respect to physical or genetic parameters. Several techniques commonly used for physical characterization of plasmids were applied in this project to study the plasmids of *Acinetobacter* (8W-1), *Aeromonas* (38W-HC10-1) and especially *Flavobacterium* (8W-2). One of the simpler methods used was the determination of molecular weights of intact plasmids by relative mobility in agarose gels. Digestion of purified plasmids with restriction endonucleases was used for physical characterization by providing molecular weight estimates, and comparisons of restriction fragment patterns with a known degradative plasmid (TOL) also enabled estimation of homology (Heinaru *et al.*, 1978). The genetic character also was determined by hybridization with a ^{32}P-TOL probe prepared by a standard method (Southern, 1979).

Plasmid instability *in vitro* has been reported previously (Wheatcroft and Williams, 1981), but no explanations for the phenomenon were offered. In this project, the physical and structural basis for the unique instability demonstrated by *Flavobacterium* (8W-2) plasmids was explored in some detail. Various pre- and post-isolation treatments were employed, including proteinase incubation and U.V. nicking of covalently closed circular plasmid DNA.

Attempts were made to correlate the observed results with current theories regarding plasmid associations with RNA (Hogrefe and Friedrich, 1984), protein (Rouviere-Yaniv and Yaniv, 1979; Clewell and Helinski, 1970) and chromosomal DNA (Miller and Kline, 1979).

Conjugation and transformation experiments were used to attempt genetic characterization of *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) plasmids. The acquisition of new phenotypes by plasmid recipients is a standard method for determining, at least in part, the genetic content of the plasmids. However, many unsolved problems, both technical and genetic, prevented success with these plasmid transfer studies.

5.2 Methods and Materials

5.2.1 Characterization Techniques

Molecular Weight Determinations

Electrophoresis in agarose gels was used to estimate the molecular weights of intact plasmids. The following plasmids were used as molecular weight markers: R478 (166 Mdal), R27 (112 Mdal) (both strains supplied by D.Taylor, Dept. Medical Microbiology, University of Alberta); TOL (78 Mdal, from strain ATCC 33015); pJ53-R307 (33 Mdal, strain supplied by J.Brunton, Dept. Medical Microbiology, University of Alberta); and pXT210 (9 Mdal) and pBR322 (2.6 Mdal) (both strains supplied by K.Roy, Dept. Microbiology, University of Alberta). TEB buffer (0.05M Tris - 1mM Na_2EDTA - 0.05M boric acid) was commonly used for electrophoresis, but since plasmids from *Acinetobacter* (8W-1) were obscured by chromosomal material in this system, they were electrophoresed in TEAS buffer (0.5M Tris - 10mM Na_2EDTA - 0.1M sodium acetate - 0.9M NaCl).

Relative mobilities of the molecular weight markers were measured from photographs of agarose gels, and the values were plotted on semi-logarithmic graphs. From these graphs, the apparent molecular weights of the plasmids were estimated, using relative mobility.

The standard electrophoresis system employed 0.7% agarose gels. In some cases, gels of 0.5% or 1.2% were used to estimate the relative mobility of plasmids.

In one experiment, a vertical agarose gel was electrophoresed a standard length of time, removed from the gel apparatus, and stained with ethidium bromide (EtBr). The gel was then irradiated for 3 minutes on a U.V. transilluminator (Ultra-Violet Products, Inc., CA), photographed, and placed in a horizontal electrophoresis chamber with TEB buffer for further electrophoresis. The gel was run an appropriate length of time, then re-photographed. The movement of the plasmid bands after this "U.V. nicking" procedure was intended to be a measure of the plasmids' tertiary structure.

Restriction Digests

Plasmid DNA for restriction endonuclease digestions was obtained by recovery from preparative agarose gels, as described below, or from fractions of sucrose density gradient centrifugation preparations (Chapter 3). The sucrose fractions were used directly for restriction analysis without further purification, although they often contained more than one plasmid species.

Isolation of DNA from Preparative Agarose Gels

Since *Acinetobacter* (8W-1) and *Flavobacterium* (8W-2) contained multiple plasmids, the use of dye buoyant density (CsCl) centrifugation or rate zonal (sucrose) centrifugation was inappropriate for isolating single plasmid species for individual studies. Therefore, plasmid DNA for restriction digests was usually prepared in one of four ways: electroelution, thermal disruption of low melting temperature agarose, physical/mechanical disruption by the "freeze-squeeze" technique, or by maceration.

To effect these techniques, plasmid preparations (made by the modified Casse procedure or the sucrose gradient technique) were electrophoresed in 0.7% agarose LE (SeaKem) or 0.8 to 1.0% low melting temperature agarose (Type VII, Sigma). (Alternatively, horizontal gels of 0.7% SeaKem agarose were prepared with 0.8% Type VII agarose inserts and samples of plasmid DNA were electrophoresed into the Type VII agarose.) Bands were visualized by staining the gel with EtBr and irradiating with ultraviolet light, then were carefully excised from the gel.

For electroelution, gel strips were placed in freshly boiled dialysis tubing containing a minimum volume of sterile TEB electrophoresis buffer. The tubing was closed with Spectropore® closures (Millipore), then placed perpendicular to current flow in a horizontal electrophoresis chamber containing TEB sufficient to surround but not completely submerge the tubing. A voltage gradient was applied across the chamber (usually 100 v) for 2 to 10 hr. The apparatus and samples were protected from light during the electrophoresis. Migration of the EtBr-stained DNA from the agarose strips was monitored with a hand-held U.V. source (Mineralite, UltraViolet Products, CA). When the majority of the fluorescence had moved out of the agarose, the polarity of the current was reversed for 2 min to remove the DNA from the tubing wall. The buffer in the tubing was removed and pooled with minimal volume TEB washings of the tubing contents. This DNA solution was then treated to remove trace amounts of agarose and the EtBr, and to concentrate the DNA, usually by phenol/ chloroform extraction and iso-propanol or ethanol precipitation.

The use of low melting temperature agarose gels permitted controlled thermal disruption of excised plasmid DNA bands (Maniatis *et al.*, 1982). The individual agarose bands were placed in 1 to 5 volumes of 50/20 TE buffer in sterile tubes and heated at 65°C just until the gel had melted. The solution was immediately extracted twice with 50/20 TE-saturated melted crystalline ultrapure phenol (Bethesda Research Laboratories), then twice with phenol:chloroform (1:1), and twice with chloroform to remove the agarose, microfuging between extractions. The aqueous phase was precipitated at -20°C in ethanol and collected for further centrifugation or for further extraction and purification.

Physical/mechanical disruption of gels by the "freeze-squeeze" method (Smith, 1980) was used to recover plasmid DNA from preparative agarose gels. Excised agarose bands were placed in microfuge tubes, rapidly frozen in dry ice/ethanol and thawed at 37°C (two cycles), then centrifuged 5 min at maximum speed in a microfuge (11,000 x g). The supernatant was recovered, while the pelleted agarose was again frozen, thawed, and re-centrifuged. The pooled supernatants were concentrated by repeated extraction with n-butanol, with occasional centrifugation to separate the phases. The final concentrated supernatant was used for restriction endonuclease digestion analysis.

An alternate method used maceration of excised agarose strips, which were frozen at -20°C. While frozen, the strips were physically crushed between two sheets of Parafilm®, using finger pressure. The extruded liquid was collected, extracted with phenol: chloroform (1:1) to purify, and extracted with n-butanol to concentrate the DNA.

Restriction endonuclease digests were performed according to suppliers' recommendations, using appropriate buffers and incubating at 37°C. DNA from phage λ was often included as a positive digestion control. The digestions were stopped either by heating to 65°C for 10 min or by adding a 50% glycerol - 100mM Na_2EDTA - 1% SDS - bromphenol blue mixture. Digestion mixtures of DNA recovered from Type VII agarose sometimes included "nuclease-free" bovine serum albumin (BSA; Bethesda Research Laboratories, courtesy K.Roy, Dept. Microbiology, University of Alberta), as recommended by Parker and Seed (1980) to overcome the inhibitory effect of agarose. All digests were loaded onto vertical agarose gels, (usually in TEB buffer), with appropriate size markers (usually HindIII restricted phage λ DNA).

Measurements of relative mobilities of standard molecular weight markers were made from photographs of the agarose gels, and values were plotted on semi-logarithmic paper. Apparent molecular weights of digest fragments from plasmids were interpolated from the standard graphs, (Southern, 1979). Fragment sizes were summed to give an approximate molecular weight value for the intact plasmid.

5.2.2 Homology with the TOL plasmid pWWO

To study homology between the *Aeromonas* (38W-HC10-1) plasmid (p38W-1) and TOL (pWW0), an agarose gel of restriction endonuclease digest fragments from p38W-1 and TOL was blotted onto nitrocellulose by a modification to the method of Southern (1979). ^{32}P-nick labelled TOL (pWW0) DNA was used as a probe. All manipulations were performed in the laboratory of, and supervised by K.L.Roy (Dept. Microbiology, University of Alberta). The methods used are outlined below. Plastic gloves were worn throughout the procedure.

Southern Transfer

Nitrocellulose filter paper (Schleicher and Schuell) was cut 1cm larger on each side than the trimmed agarose gel, then thoroughly wetted with distilled water. The nitrocellulose was allowed to soak in 20x SSC (1x SSC: 0.15M NaCl - 0.015M sodium citrate, pH 7.0) until transfer.

The agarose gel containing the plasmid digests was prepared by depurinating the DNA with an acid treatment of 10 to 15 min in 0.25M HCl. The DNA was then denatured *in situ* by washing the gel in 0.15M NaCl - 0.5M NaOH for 5 min, then soaking the gel in fresh alkaline solution twice, for 15 min each time. The gel was neutralized in 3M NaCl - 0.5M Tris (pH 7.4), soaking twice for 15 min each time in the neutralizing solution.

Blotting paper (Whatman #3) was draped over a reservoir of 20x SSC to act as a wick. Two pieces of blotting paper just larger than the gel were soaked in 20x SSC and placed on the wick. The gel was placed on top of these papers, surrounded by plexiglass spacers the same thickness as the gel, and plastic wrap. The prepared nitrocellulose was then placed on top of the gel, touching the entire surface of the gel and resting on the plastic wrap-covered spacers. A piece of blotting paper the same size as the gel was centered over the gel, then weighted with a stack of paper towels and a lead weight for overnight transfer at room temperature.

Upon disassembly, the position of the agarose gel on the nitrocellulose was carefully marked and the nitrocellulose was washed carefully with 2x SSC. The DNA was then immobilized by baking the filter in a vacuum oven at 80°C for 2 hr, and stored at room temperature until needed for hybridization.

^{32}P-Nick Translation of TOL (pWW0) Probe

TOL (pWW0) DNA from *P. putida* (ATCC 33015) prepared by sucrose density gradient centrifugation was used as the probe for TOL homology studies. Approximately 100µL of plasmid DNA solution (≃ 3µg) was ethanol precipitated, washed with 95% ethanol, and air dried. The DNA was resuspended in 41µL distilled water plus 25µL 4x nick translation buffer (4x NT buffer: 0.2M Tris, pH 7.8 - 0.02M Na_2EDTA - 0.01M dithiothreitol - 200µg/mL bovine serum albumin) plus 2µL each of dCTP, dGTP and dTTP (Mannheim-Boehringer) plus 20µL ^{32}P-dATP (3000 Ci/mmol; New England Nuclear); this mixture was preincubated for 10 min at 15°C. Then 7µL of DNA polymerase I (0.3 units/µL; Bethesda Research Laboratories) and 1µL of diluted DN'ase (BioLabs) were added to the solution and incubated for 1 hr at 15°C. Nick translation was stopped by adding 5µL of 0.25M Na_2EDTA containing 2% xylene cyanol. The radioactive solution was passed through a miniature (approximately 5mL) Sephadex G25 column, eluting with 10mM Tris - 1mM Na_2EDTA - 0.1% SDS. The nick translated DNA eluted after the first column volume, and was separated from the unused deoxynucleotides, using a hand-held geiger counter (Mini-Instruments, England) to monitor elution. The nick translated DNA was stored at -20°C until needed for hybridization.

Hybridization

The nitrocellulose filter with transferred DNA was placed in a sealable plastic bag and wetted with 5mL of 5x SSC for 20 min to rehydrate. Then 4mL of Denhardt's reagent (0.02% Ficoll - 0.02% polyvinylpyrrolidone - 0.02% bovine serum albumin in 3x SSC) and approximately 80µL (≃ 200µg) of *E. coli* chromosomal DNA (denatured at 90°C for 5min and diluted with 400µL distilled water plus 5µL 0.25M Na_2EDTA; used as competitor DNA to reduce non-specific hybridization) were added and allowed to hybridize at 65°C for about 1 hr.

Immediately prior to use, the ^{32}P probe DNA was denatured by heating to 90°C for 5 min, then added to the prehybridized nitrocellulose filter and sealed. Hybridization was allowed to occur with mixing at 65°C overnight.

The hybridized filter was then washed under medium stringency conditions: washed twice in 2x SSC plus 0.1% SDS at 65°C for 15 min; washed three times in 0.2x SSC at 65°C for 20 to 30 min, allowing the last wash to cool to room temperature overnight; then washed once in 0.2x SSC at 65°C for 1 hr.

The hybridized filter was then placed on a piece of Kodak X-ray film which was exposed for 24 hr at -90°C and developed conventionally. This autoradiogram was compared with a photograph of the original agarose gel to determine the degree of homology of the restriction endonuclease fragments of p38W-1 with TOL (pWW0).

5.2.3 Plasmids of *Flavobacterium* (8W-2)

The apparent instability of the *Flavobacterium* (8W-2) plasmids was investigated using a variety of techniques, including procedural changes to the modified Casse technique and various post-isolation treatments. The following modifications were used separately or in combinations to explore the nature of the *Flavobacterium* (8W-2) plasmids.

1. Prior to lysis, cells were heated to 60°C, 70°C or 80°C for 15 min, chilled, and used immediately in plasmid preparations.

2. Lysis was accomplished in the non-ionic detergent Triton X-100 by a modification to the method of Clewell and Helinski (1970) (Chapter 3). Plasmid yield and stability were compared with the standard lysis mixture using 4% SDS (an ionic detergent).
3. As a variation of the modified Casse procedure, the phenol extraction step was omitted. Rather, the salt-flocculated solution was centrifuged at 4°C for 10 min at 12,000 rpm (17,300 x g) and the supernatant was ethanol precipitated to yield plasmid DNA.
4. After overnight ethanol precipitation, the harvested, dissolved plasmid DNA was heated to 37°C, 60°C, 70°C or 80°C for 10 min, then mixed with loading buffer and applied to agarose gels.
5. Different loading buffers were compared: 16% Ficoll in 50/20 TE plus Orange G; 33% glycerol in 50/20 TE plus bromphenol blue; and 33% glycerol plus 7% SDS in 50/20 TE plus bromphenol blue.
6. After dissolution in 10/4 TE, harvested plasmid DNA was incubated with autodigested Pronase® at 37°C for 15 min.
7. Different temperatures were used to store isolated plasmids, ranging from 4°C to -70°C. Aliquots of plasmid DNA were stored varying lengths of time (hours to months) and were periodically thawed at 22°C, 37°C or 65°C and assayed for plasmid content by agarose gel electrophoresis.

8. β-mercaptoethanol (BME) or dithiothreitol (DTT) were added in trace amounts to plasmid preparations either immediately after cell lysis, or during dissolution of harvested plasmid DNA, or as a supplement to the loading buffer. Yields and apparent stability of the plasmids were compared with preparations not receiving reducing agents.
9. Harvested plasmid DNA was dissolved in dilute alkali (0.1mM NaOH in 10/4 TE). Alternatively, buffers of different pH were used to dissolve the harvested plasmids.

5.2.4 Conjugation and Transformation

Conjugation Procedures

The liquid culture mating procedure of Connors and Barnsley (1982) was modified as follows. The donor *Flavobacterium* (BW-2) was grown in STCS + 10μg $HgCl_2$/mL at 24°C overnight to an approximate optical density of 1 at 600 nm. The recipient *P. putida* (ATCC 33015) was grown in TCS at 30°C overnight. Different proportions of the donor and recipient cultures were mixed together (from 1:1 to 10:1, donor:recipient culture volumes) and allowed to incubate undisturbed for 1 hr at 22°C. The cells were then harvested in a benchtop centrifuge, washed with 1mL of sterile 2.5% NaCl and diluted in sterile phosphate buffer. The dilutions were plated onto selective plates: for example, PCA + 100μg $CdCl_2$/mL overlaid with 0.5mL of a sterile solution of 20mg

$HgCl_2$/mL. These plates were incubated at 30°C and scored for growth.

In a second attempt at conjugal transfer in liquid medium, a chromosomal rifampicin resistant *Pseudomonas* strain (DT82, supplied by D.Taylor, Dept. Medical Microbiology, University of Alberta) was used as a recipient and grown overnight at 24°C in TCS broth. The donor *Flavobacterium* (8W-2) was grown overnight at 24°C in STCS. Two millilitres of each culture were mixed together and incubated, stationary, for 30 min at 22°C, then placed at 28°C for 3 hr to allow for phenotype expression. Finally, 0.1mL of cell suspension was plated onto selective PCA plates containing streptomycin plus rifampicin, incubated overnight at 28°C, and scored for growth. Inoculation of the parental strains on the selective plates showed no growth.

A third method for mating *Flavobacterium* (8W-2) by solid medium conjugation was suggested by D.Taylor (Dept. Medical Microbiology, University of Alberta). Isolate 20S-8 [a novobiocin resistant *Pseudomonas* sp. capable of degrading methylnaphthalenes (Chapter 2), but apparently lacking a plasmid] was used as a recipient and grown for 48 hr at 22°C in STCS. Two donors, *Aeromonas* (38W-HC10-1) and *Flavobacterium* (8W-2), were grown 48 hr at 22°C in STCS plus tetracycline, or 50µg $CdCl_2$/mL + streptomycin, respectively. Aliquots of 0.15mL each of a donor culture plus the recipient culture were plated onto BMA and incubated overnight at 28°C. The plates were washed with sterile STCS and the cells plated on selective media: kanamycin + novobiocin for *Aeromonas* (38W-HC10-1) plus isolate 20S-8; and streptomycin + novobiocin for *Flavobacterium* (8W-2) plus isolate 20S-8. Control inoculations of the parental strains were also performed. The same experiment was repeated using the rifampicin resistant *Pseudomonas* strain DT82 as a recipient.

Transformation Procedures

DNA for transformation was prepared by two different methods. *Flavobacterium* (8W-2) plasmid DNA excised from agarose gels was "freeze-squeezed". Alternatively, sucrose gradient fractions prepared by the method of Wheatcroft and Williams (1981)(Chapter 3) were used.

A technique for transforming *E. coli* (Maniatis *et al*., 1982) was used with recipient *Acinetobacter* (8W-1) and plasmid DNA from *Aeromonas* (38W-HC10-1), *Flavobacterium* (8W-2) or pBR322 prepared by sucrose gradient centrifugation. *Acinetobacter* (8W-1) was grown in 30mL STCS overnight at 24°C, harvested, and resuspended in one-half volume cold sterile 50mM $CaCl_2$ - 10mM Tris (pH 8) and incubated 10 min on ice. The cells were again harvested, resuspended in one-fifth volume of the $CaCl_2$ - Tris buffer, and incubated 5 min on ice. The putatively competent cells were stored overnight at 4°C in 200µL aliquots, then used for transformation by mixing an aliquot with 50µL of a sucrose fraction containing intact *Aeromonas* (38W-HC10-1), *Flavobacterium* (8W-2) or pBR322 plasmids, incubating on ice for 30 min. The cells were heat shocked at 42°C for 3 min, then diluted with 1mL of STCS broth and incubated without shaking at 28°C for 30 min. The cells were plated onto BMA, sprayed with fluorene or DBT and incubated at 26°C to score for colored products or cleared zones.

To transform "cured" variants of *P. putida* (ATCC 33015), recipients were prepared by serially transferring *P. putida* (ATCC 33015) in a minimal salts - benzoate medium and screening for m-tol$^-$ variants by replica plating on m-toluate plates. None of the 40 m-tol$^-$ variants screened for plasmids proved to be completely cured, but variants #8, #12 and #13 showed a deletion in the TOL plasmid and were chosen as the recipients. The procedure of Mercer and Loutit (1979) was modified for use as follows. An aliquot of overnight growth of *P. putida* (ATCC 33015) variants #8, #12 or #13 on PCA was used to inoculate 30mL of prewarmed TCS broth which was incubated 3 hr at 30°C to achieve log phase growth. The cells were harvested, resuspended in one-half volume cold sterile 0.15mM $MgCl_2$ and incubated on ice for 5 min. The cells were harvested again, resuspended in one-half volume 0.15mM $MgCl_2$, and incubated on ice for 20 min. These putatively competent cells were harvested, resuspended in one-tenth volume 0.15mM $MgCl_2$ and divided into 200µL aliquots. Ten to fifty microlitres of DNA solution were added to each aliquot of cells and incubated on ice for 60 min. A heat pulse of 37°C for 3 min with swirling was followed by incubation on ice for 5 min, then 0.5mL of TCS broth was added and the suspension was incubated at 37°C for 3.5 hr. The cells were plated, using: PCA ± streptomycin or $HgCl_2$ or sprayed with fluorene or DBT; or minimal medium + 5mM o-phthalate; or minimal medium saturated with 1-hydroxy-2-naphthoate (<5mM).

The technique of Christopher *et al.* (1981) was modified for use with *Flavobacterium* (8W-2) plasmid DNA (prepared by the modified Casse method) and recipient *P. putida* (ATCC 33015) benzoate-"cured" variant #13. The recipient was grown at 30°C in TCS broth overnight, then 1mL was transferred to 30mL fresh TCS broth and incubated at 30°C with shaking for 3 hr to produce a log phase culture. The recipient cells were harvested in sterile Teflon® screw cap tubes, washed with 20mL of sterile Buffer I (MOPS 10mM - KCl 10mM - $MnCl_2$ 150mM) and resuspended in 20 mL of sterile Buffer II (MOPS 100mM - $CaCl_2$ 100mM - KCl 10mM). In both buffers, KCl and $MnCl_2$ replaced $RbCl_2$ and $MgCl_2$ in the method of Christopher *et al.* (1981), using the substitution recommendations of Hanahan (1983). The cells in Buffer II were incubated on ice for 30 min, then harvested and resuspended in Buffer II, using one-tenth the original culture volume. Two-tenths millilitre of this cell suspension was added to approximately 35μL plasmid DNA solution in 10/4 TE buffer, and incubated on ice for 45 min. Without mixing the suspension, the cells were heat shocked at 42°C for 2 to 3 min, then diluted with 3mL of prewarmed

TCS broth and incubated at 30°C for 90 min, after which time they were plated onto appropriate media. Both selective (i.e. metal- or antibiotic-containing; and minimal agar sprayed with PAH as sole carbon source) and differential (i.e. PCA sprayed with PAH) plates were used, and incubated at 30°C. Selective plates were scored for growth while differential plates were scored for colored metabolite production or clearing of the PAH layer.

As a variation on this technique, prior to being made competent, the *Pseudomonas* sp. recipient cells were grown overnight at 30°C, then transferred to 42°C for 3 hr to reduce the production of endogenous restriction endonucleases (Nakazawa, 1978).

5.3 Results and Discussion

5.3.1 Characterization Techniques

Molecular Weight Determinations

Intact plasmids of known size were used as molecular weight markers to estimate the apparent molecular weights of plasmids from *Acinetobacter* (8W-1), *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1). Plate 5.1 shows the relative mobilities and estimated molecular weights of the 5 major plasmids (pF1 to pF5) in *Flavobacterium* (8W-2). Numerous measurements were made using different gels, and apparent

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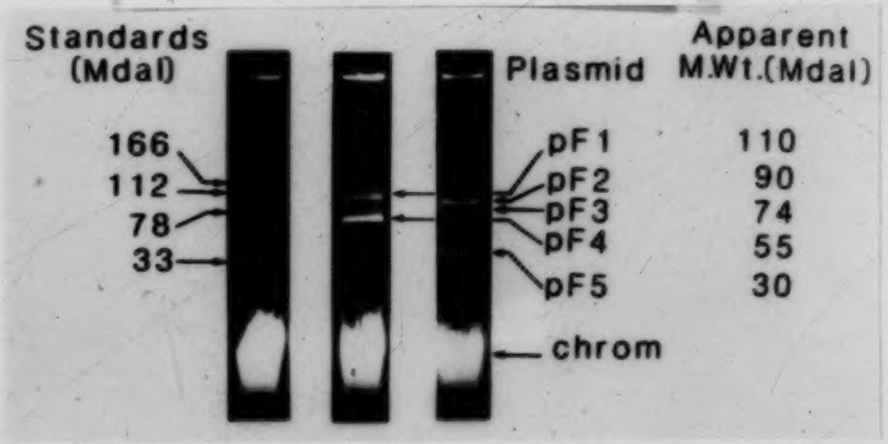

Standards (Mdal)
166
112
78
33
Plasmid
Apparent M.Wt.(Mdal)
pF1 110
pF2 90
pF3 74
pF4 55
pF5 30
chrom

Plate 5.1 Molecular weight estimations of 5 major plasmids in *Flavobacterium* (8W-2) wild type and variants. Molecular weight marker plasmids: R478 166Mdal; R27 112Mdal; TOL (pWWO 78Mdal; pJ53-R307 33Mdal; chrom = chromosomal material

molecular weights were averaged to allow presentation of the mean molecular weight (Mdal) ± one standard deviation with sample size n: pF-1 110 ± 5 (n=14); pF-2 90 ± 3 (n=8); pF-3 74 ± 4 (n=25); pF-4 55 ±4 (n=13); pF-5 30 ± 3 (n=10). Determinations of molecular weights for the plasmids in *Acinetobacter* (8W-1) and *Aeromonas* (38W-HC10-1) were not as precise because these isolates were not the primary focus of the thesis. Plasmids in *Acinetobacter* (8W-1) were calculated to have the following molecular weights: p8W1-1 17 Mdal; p8W1-2 9 Mdal; p8W1-3 7 Mdal; and p8W1-4 5 Mdal, as determined in TEAS buffer. Although the molecular weight of the single plasmid p38W-1 in *Aeromonas* (38W-HC10-1) was difficult to estimate from the relative mobility of the intact plasmid because of its large size, it was calculated to be 122 ± 9 Mdal (n=4), using TEB buffer.

Preparations of *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1) plasmids were subjected to electrophoresis in TEAS buffer at least once to check for extra plasmids co-migrating with chromosomal material in TEB buffer. No extra plasmids were found. Similarly, following the method of Mickel *et al*. (1977), plasmid preparations of *Aeromonas* (38W-HC10-1) and *Flavobacterium* (8W-2) were run in a 1.2% agarose gel to determine whether there were any plasmid species co-migrating with chromosomal material in the standard 0.7% agarose gels, as had been observed by Kiyohara *et al*. (1983). No extra plasmids were observed, leading to the conclusion that electrophoresis in 0.7% agarose with TEB buffer was adequate for visualizing all plasmids in *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1).

Restriction Endonuclease Digests

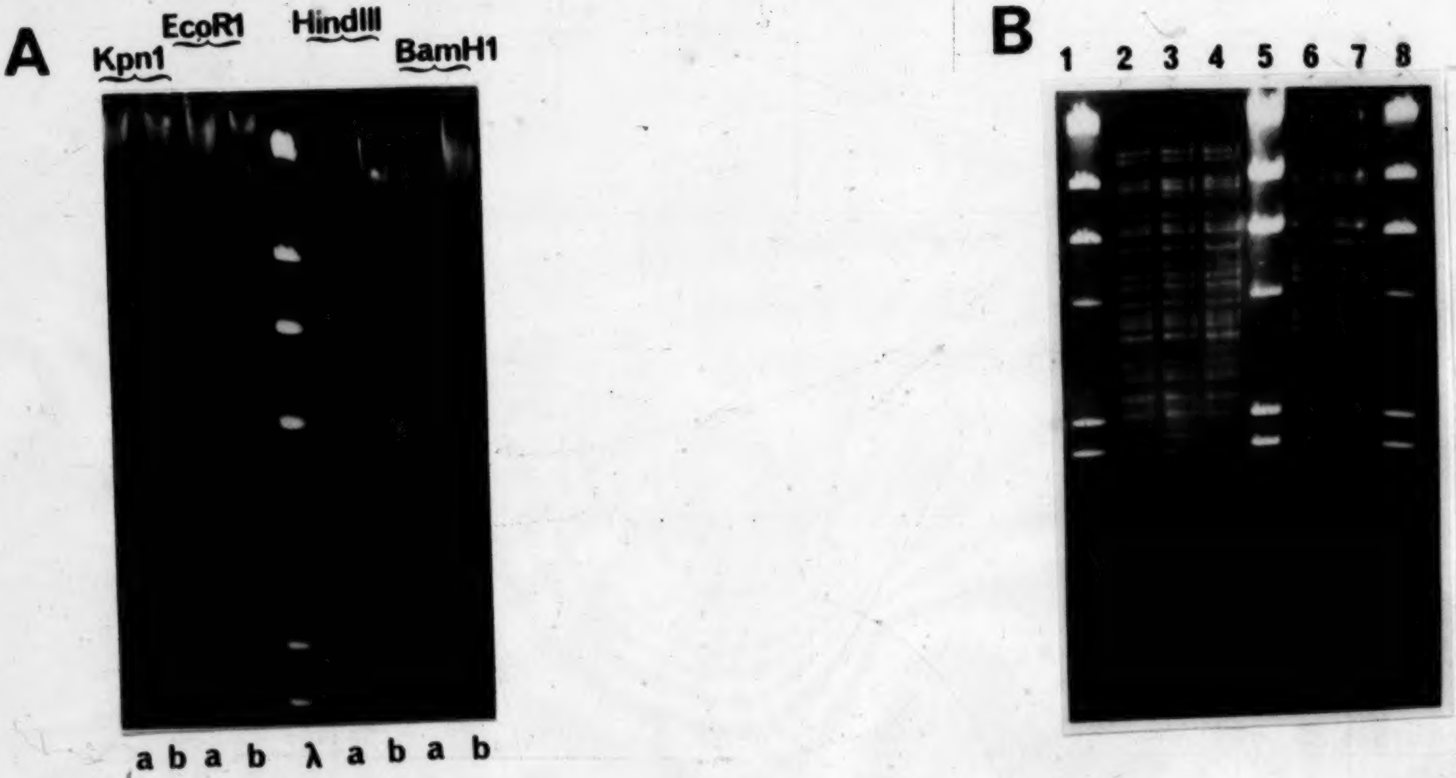
Restriction digests of plasmids were used for three purposes: to estimate the size of p38W-1 [the single plasmid in *Aeromonas* (38W-HC10-1)]; to identify similarity of patterns between the wild type strain and phenotypic variants in both *Flavobacterium* (8W-2) and *Aeromonas* (38W-HC10-1); and to observe homology between p38W-1 and the TOL (pWW0) plasmid.

None of the methods used for recovery of plasmids from preparative agarose gels was particularly successful, and, as predicted by Smith (1980), electroelution of large plasmids was very slow and incomplete. Recovery by compression of frozen agarose slices either by centrifugation or the "freeze-squeeze" method was incomplete, since observation of the residual agarose revealed high levels of EtBr fluorescence, indicative of the presence of DNA. Thermal disruption of Type VII agarose proved to be the most effective means of recovering DNA from preparative gels. However, despite repeated extractions, the recovered DNA appeared to be contaminated with residual agarose because it could not be cut by restriction enzymes. Phage λ DNA included in some experiments as a positive control for recovery and restriction was recovered in higher proportions than plasmid DNA, undoubtedly because of its smaller size. However, to effect complete digestion of the λ DNA, the inclusion of "nuclease free" BSA in the digest mixture was necessary. This added component apparently overcomes the inhibitory effects of agarose on some restriction endonucleases (Parker and Seed, 1980). Reagent grade BSA (Sigma) was not adequate for restriction analyses.

Therefore, because of the problems associated with both incomplete recovery of large plasmids from preparative agarose gels, and with subsequent purification adequate to allow restriction endonuclease digestion, few digests were successful. The best digests were accomplished with material from sucrose gradient fractions, which required no further purification before digestion. However, because of the presence of multiple plasmids in *Acinetobacter* (8W-1) and *Flavobacterium* (8W-2), this method was particularly successful only for *Aeromonas* (38W-HC10-1), with its single plasmid p38W-1.

Plasmid p38W-1 from *Aeromonas* (38W-HC10-1) and TOL (pWW0) digested with Kpn1, EcoR1, HindIII and BamH1 are shown in Plate 5.2 (Panel A). Kpn1 and EcoR1 enzymes did not restrict the plasmid DNA. There is no similarity between p38W-1 and TOL (pWW0) in the pattern of fragments generated with HindIII or BamH1. Using HindIII, the digestion patterns of 5 phenotypic variants of *Aeromonas* (38W-HC10-1) can be compared (Plate 5.2, Panel B). There are no apparent differences in HindIII restriction patterns among the 5 variants, and comparisons with digested *Aeromonas*

Plate 5.2 Restriction endonuclease digests of TOL (pWWO) and p38W-1 from *Aeromonas* (38W-HC10-1) wild type and phenotypic variants.



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Panel A: a: TOL (pWWO) plasmid from *P. putida* (ATCC 33015); b: p38W-1 plasmid from *Aeromonas* (38W-HC10-1) wild type; λ: HindIII digest of phage λ DNA as molecular weight markers (Mdal): 7.1, 2.9, 2.0, 1.3, 0.7, 0.6.

Panel B: HindIII digests of plasmids from *Aeromonas* (38W-HC10-1) phenotypic variants: Lanes 1,5,8: HindIII digest of phage λ DNA as molecular weight markers (Mdal): 7.1, 2.9, 2.0, 1.3, 0.7, 0.6. Lane 2: variant N35; Lane 3: variant N34; Lane 4: variant N33; Lane 6: variant N29; Lane 7: variant N28.

(38W-HC10-1) wild type plasmid (p38W-1) did not reveal pattern differences (data not presented). Relatively small plasmid deletions should have been detected by this method, considering the frequency of restriction, but the lack of change in fragment patterns suggests that plasmid deletions are not responsible for the change in phenotype. There is still the possibility that point mutations or very small deletions in the plasmid, which could not be detected by this method, are responsible for phenotypic variation.

Using digest gels such as those shown in Plate 5.2, an estimated molecular weight for p38W-1 based on digestion fragments was calculated to be 125 ± 12 Mdal (n=7). This correlates quite well with the molecular weight of 122 ± 9 Mdal estimated by the relative mobility of the intact plasmid.

Great difficulty was encountered in attempting to digest isolated *Flavobacterium* (8W-2) plasmid DNA with restriction enzymes. As previously discussed, recovery of individual plasmids from agarose gels was incomplete and did not provide sufficiently pure preparations for restriction analysis. Therefore, sucrose gradient fractions of *Flavobacterium* (8W-2) plasmid mixtures were restricted with the following endonucleases: HindIII, EcoR1, ClaI, BamH1, HpaI, BglII and XbaI; only HindIII and EcoR1 gave reasonably good digestion patterns. In some digestion mixtures, phage λ DNA was incorporated as a positive internal restriction indicator. In several cases, the λ DNA digested as expected, while the *Flavobacterium* (8W-2) plasmid DNA presented an indistinct pattern of fragments with background smearing (data not presented), which may have been due to chromosomal contamination of the sucrose gradient fraction. These problems with restriction of *Flavobacterium* (8W-2) plasmids hampered comparison of fragment patterns between phenotypic variants, and the presence of multiple plasmids complicated molecular weight estimations. No reproducible values were calculated for total plasmid molecular weight. Restriction bands common to both the wild type and phenotypic variants were observed in each digest regardless of the endonuclease used (data not presented), which substantiates the assumption that the plasmids observed in the phenotypic variants are derived from the plasmids of the wild type *Flavobacterium* (8W-2). No similarity between *Flavobacterium* (8W-2) digestion patterns and TOL (pWW0) patterns was observed.

Interestingly, in the most sucessful endonuclease digests with *Flavobacterium* (8W-2) and EcoR1 or HindIII, several bands were observed which were much more intense than the majority of bands (Plate 5.3). It is assumed that these intense bands contain large amounts of DNA of the same size, possibly representing "repeats" of DNA, which may be found within the same plasmid in a strain, or repeated in different plasmids within a strain. Whether or not these supposed repeats represent duplicated genes is open to speculation.

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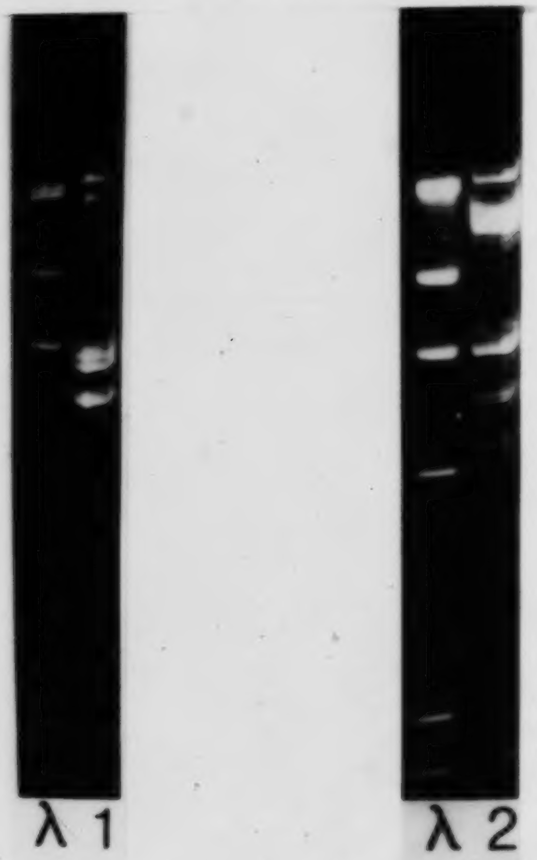

Plate 5.3 Restriction endonuclease digests of plasmids in *Flavobacterium* (8W-2) variant Maint, showing intense bands. λ = HindIII digest of phage λ DNA as molecular weight markers (Mdal): 7.1, 2.9, 2.0, 1.3, 0.7, 0.6; Lane 1 = EcoR1 digest; Lane 2 = HindIII digest.

No attempts to digest plasmids of *Acinetobacter* (8W-1) with restriction enzymes were successful, and no further efforts were directed towards this isolate.

5.3.2 Homology with TOL

The use of Southern transfer and ^{32}P-TOL hybridization techniques permitted observation of homology between plasmid p38W-1 from *Aeromonas* (38W-HC10-1) and the TOL (pWW0) plasmid. ^{32}P-TOL was used to probe a restriction digest of *P. putida* (ATCC 33015) and *Aeromonas* (38W-HC10-1) (Plate 5.2, Panel A). The autoradiogram of that probed agarose gel is shown in Plate 5.4. The probe hybridized perfectly with its isogenic restriction digests, and with its chromosomal DNA and unrestricted plasmid band. However, there is no indication of hybridization to any portion of the *Aeromonas* (38W-HC10-1) digest, whether plasmid or chromosomal, under medium stringency wash conditions. This observation, in combination with the differences in restriction patterns of the two plasmids (Plate 5.2, Panel A) suggests that there is no significant homology between plasmid p38W-1 and TOL (pWW0). The lack of homology is not surprizing, since other degradative plasmids encoding related functions (e.g. SAL and NAH) have shown sparing or no homology with TOL (Heinaru *et al.*, 1978). For this reason, and other, technical reasons, hybridization of ^{32}P-TOL with plasmids of *Acinetobacter* (8W-1) or *Flavobacterium* (8W-2) was not attempted.

Plate 5.4 Contact print (negative) of autoradiogram of TOL (pWWO) and p38W-1 plasmid digests probed with ³²P-TOL. (Photograph of original agarose gel is shown in Figure 5.2 Panel A.)

a: p38W-1 plasmid from *Aeromonas* (38W-HC10-1)
b: TOL (pWW0) plasmid from *P. putida* (ATCC 33015)
Lanes 1a,1b: digested with KpnI
Lanes 2a,2b: digested with EcoR1
Lane 3: HindIII digest of phage λ DNA
Lanes 4a,4b: digested with HindIII
Lanes 5a,5b: digested with BamH1

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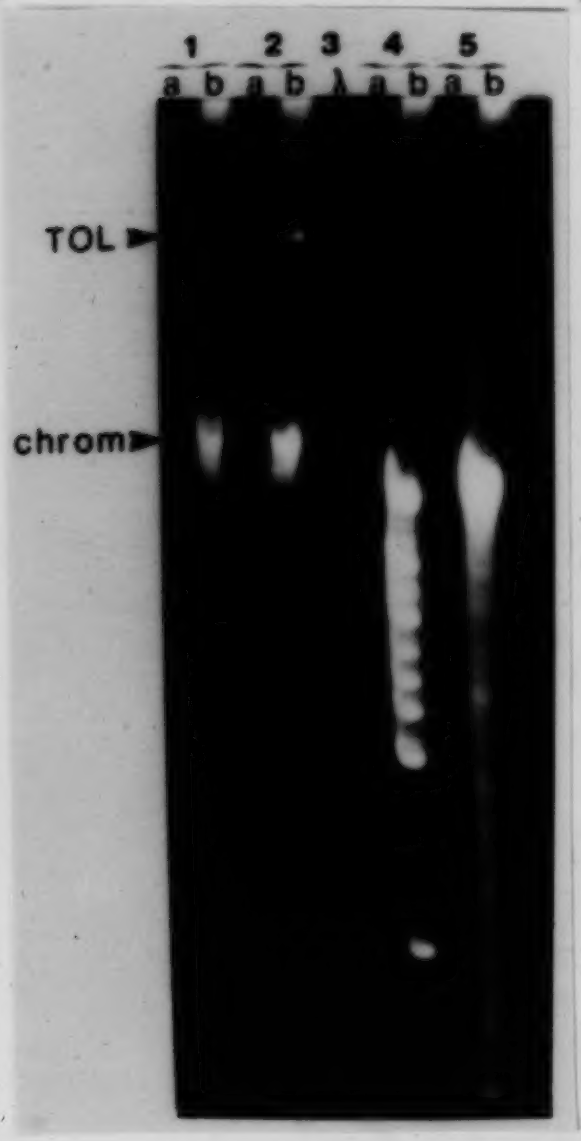
1
2
3
4
5
a
b
a
b
λ
a
b
a
b
TOL
chrom

5.3.3 Characterization of *Flavobacterium* (8W-2) Plasmids

Plasmid "instability" unique to *Flavobacterium* (8W-2)

It was observed repeatedly that the plasmids of *Flavobacterium* (8W-2) demonstrated a unique "instability" upon storage. Plasmids isolated from all other genera, whether PAH degrading or not, were stable under storage at -20°C for periods up to or exceeding one year. That is, once isolated, the purified plasmids could be visualized consistently on agarose gels, running with the same relative mobility as the day they were isolated. Eventually, of course, with repeated free-thaw cycles, or with prolonged storage at temperatures above freezing, some deterioration occurred and smearing of the plasmid bands in the agarose gels occurred. However, the plasmids of *Flavobacterium* (8W-2) could not be visualized consistently in agarose gels, regardless of the storage conditions. If an agarose gel was loaded with material taken immediately after dissolution of the ethanol-precipitated plasmids, the gel would show a plasmid profile characteristic of the particular *Flavobacterium* (8W-2) strain. However, a few hours later, an aliquot of the same preparation would show fewer plasmid bands, or no plasmids at all, or a large fluorescent smear running from the loading well to the chromosomal material. This occurred repeatedly with plasmid preparations stored at 22°C, 4°C, -20°C or -70°C, regardless of the presence of proteinase inhibitors or denaturing agents such as SDS or chloroform.

This might have indicated the presence of a highly active, contaminating DN'ase such as that observed by Blaschek and Klackik (1984), except that, upon prolonged storage over several days or weeks, aliquots of the same preparations would often demonstrate the presence of plasmids in patterns indistinguishable from the first visualization. This period of 'disappearance' of the plasmids is here referred to as 'eclipse', and an example of this phenomenon is presented in Plate 5.5. The onset of eclipse varied, but usually occurred within 2 to 8 hr of plasmid dissolution. Duration of eclipse also varied, ranging from 30 hr to greater than 72 hr. In fact, some plasmid preparations did not demonstrate plasmids again after the initial disappearance, and whether this is indicative of an extended eclipse period or is a reflection of actual loss of the plasmids is not known.

The variability of the eclipse phenomenon with respect to onset, duration and extent (i.e. all plasmids or only some in the profile disappear) delayed recognition of the phenomenon. In fact, many early preparations of *Flavobacterium* (8W-2) plasmids that were discarded because they appeared to have failed actually may have been successful, but the plasmids had entered eclipse before the first gel was run. Similarly, many plasmid preparations were discarded after the initial disappearance of the plasmids, in the belief that the plasmids had been irretrievably degraded or lost.

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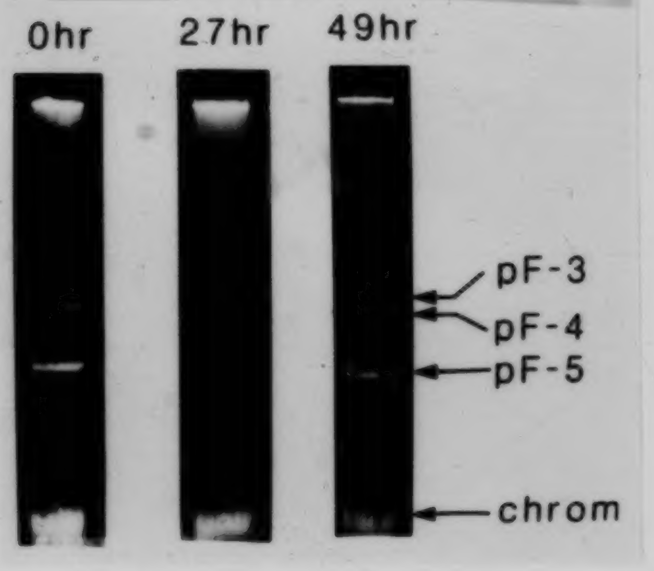
0hr
27hr
49hr
pF-3
pF-4
pF-5
chrom

Plate 5.5 Eclipse of isolated *Flavobacterium* (8W-2) plasmids stored at -20°C for 0, 27 or 49 hr.

R. Morgan (Dept. Biochemistry, University of Alberta) suggested that the unreliability of appearance of plasmid bands might be due to inadequate dissolution of the isolated plasmids after ethanol precipitation. To test this theory, 0.1mM NaOH was used to dissolve the isolated plasmids instead of the usual 10/4 TE buffer (pH 8). However, this precaution had no effect on the stability of the plasmids (data not presented). In fact, in an experiment comparing plasmid stability, aliquots of a single plasmid preparation were dissolved in 10/4 TE buffer of pH 6, pH 7, pH 8 or pH 10. These aliquots were stored at -20°C and run on agarose gels at intervals. While the pH 8 buffer (usual dissolving buffer) presented a superior plasmid profile at Time Zero, all 3 plasmids in all four solutions went into an eclipse which lasted longer than 5 d. After 8 d storage, all three plasmids in the pH 8 buffered solution had reappeared, while plasmid profiles from the other solutions were still faint and incomplete (data not shown). This would indicate that dissolution in a buffer of higher pH does not prevent eclipse; rather, the best buffer for dissolution appears to be the usual 10/4 TE buffer.

Literature references to the instability of isolated plasmids were consulted in an attempt to explain the disappearance of the *Flavobacterium* (8W-2) plasmids. A particular phenomenon studied by Helinski and others, the relaxation complex, initially appeared attractive. The relaxation complexes originally described by Clewell and

Helinski (1970) consisted of a protein associated with the ColE1 plasmid, which introduced strand-specific nicks into the supercoiled plasmid to relax it from its covalently closed circular form (CCC) to an open circular form (OC). The relaxation property of the protein was activated by the presence of SDS (at levels as low as 0.025%), by alkaline conditions (pH 12.5), by phenol treatment, by heating to 60°C for 20 min, as well as by other treatments such as incubation with EtBr; in short, by most of the conditions used in the modified Casse procedure. On the other hand, heating the cells before lysis appeared to dissociate the ColE1 complex, preventing relaxation, as did the use of non-ionic detergents for lysis, or the incubation of the isolated plasmid DNA with 1M NaCl or 0.022M β-mercaptoethanol (BME).

The significance of this relaxation is that, according to Mickel *et al.* (1977), OC plasmids larger than 14 to 16 Mdal will not enter an agarose gel, but will be retained in the loading well. The "disappearance" of the *Flavobacterium* (8W-2) plasmids might be explained if CCC forms were being transformed into OC forms during storage by the action of a relaxation complex. Failure to observe *Flavobacterium* (8W-2) plasmid DNA in CsCl-EtBr buoyant density centrifugation would also be explained by a relaxation complex sensitive to EtBr (Clewell and Helinski, 1970). Although this theory would predict the disappearance of the *Flavobacterium* (8W-2) plasmids, it cannot explain the eclipse phenomenon with reappearance of the plasmids. Regardless, a number of experiments were performed to attempt to demonstrate the role of a protein in the instability unique to the *Flavobacterium* (8W-2) plasmids.

Clewell and Helinski (1970) found that heating *E. coli* cells to 70°C for 15 min prior to lysis dissociated the ColE1 relaxation complex so that subsequently isolated plasmid DNA was stable. They also found that heating isolated ColE1 plasmids (obtained from unheated cells) to 60°C for 20 min activated the relaxation complex, and produced OC plasmids. Therefore heat "pre-treatments" of intact *Flavobacterium* (8W-2) cells and "post-treatments" of isolated plasmids were used to attempt to demonstrate stability and disappearance of plasmids, respectively. Neither heat treatment had any specific effect on plasmid stability compared with an unheated control preparation, as demonstrated in Plate 5.6, except that heating of intact cells to 80°C reduced the amount of plasmid DNA recovered from the cells, probably through premature lysis. Aliquots of the isolated plasmids were stored at 4°C, -20°C or -70°C and run on agarose gels at intervals. By the first interval (6 hr storage), the upper two plasmids (pF3 and pF4) had disappeared from all aliquots. Results from the 4°C storage condition are presented in Plate 5.6; storage at -20°C and -70°C produced identical results. After 71 hr storage, pF3 and pF4 still had not reappeared, regardless of heat treatments or storage temperatures used (data not shown);

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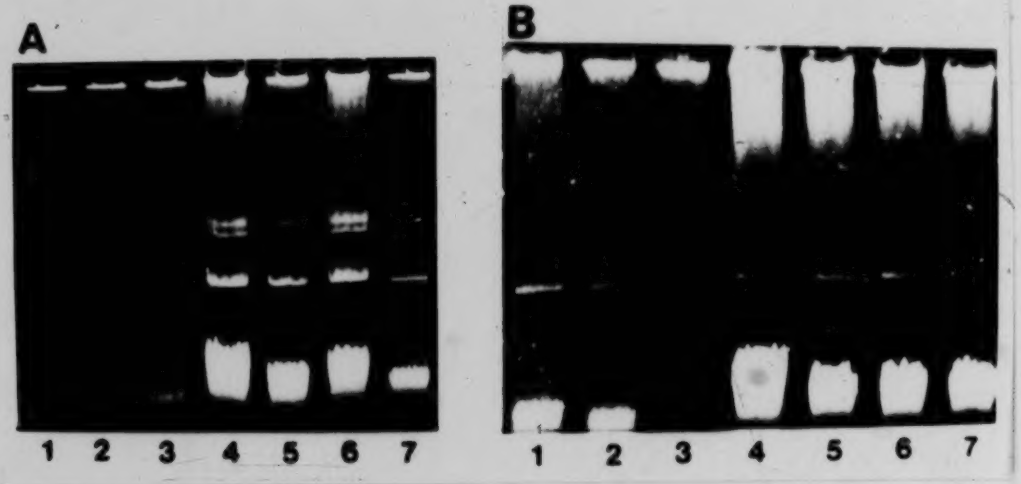

Plate 5.6 Effect of heat pre-treatment of Flavobacterium (8W-2) cells and post-treatment of isolated plasmids on plasmid stability in vitro.

Panel A: no storage of plasmids; Panel B: 6 hr storage of plasmids at 4°C

Lane 1: 60°C pre-treatment of cells; Lane 2: 70°C pre-treatment of cells; Lane 3: 80°C pre-treatment of cells; Lane 4: unheated control; Lane 5: 60°C post-treatment of plasmids; Lane 6: 70°C post-treatment of plasmids; Lane 7: 75°C post-treatment of plasmids.

the lower plasmid (pF5) however, still remained.

The use of a non-ionic detergent was recommended by Clewell and Helinski (1970) for isolating non-activated relaxation complexes of ColE1 plasmids, which otherwise could be activated by the presence of SDS. Therefore, in preparing *Flavobacterium* (8W-2) plasmids, a procedure using Triton X-100 for lysis (modified Clewell and Helinski, Chapter 3) was used instead of the modified Casse *et al.* method. As well, 3 different loading buffers containing SDS or Ficoll were tested. No plasmids were observed with the Triton X-100 lysis technique and of those plasmids isolated by the modified Casse *et al.* procedure, no effect of loading buffers ± SDS was observed.

Omission of phenol (which activated the ColE1 relaxation complex; Clewell and Helinski, 1970) from the extraction step in the modified Casse procedure did not result in *Flavobacterium* (8W-2) plasmid stability.

Similarly, incubation of *Flavobacterium* (8W-2) plasmids with Pronase®, which was found by Kupersztoch-Portnoy *et al.* (1975) to activate the relaxation complex of three R plasmids in *E. coli*, did not change the eclipse period of the *Flavobacterium* plasmids compared with an untreated plasmid preparation. The photograph in Plate 5.7 shows the eclipse of parallel plasmid preparations treated with Pronase®, or heated with loading buffer containing SDS, or resuspended in Ficoll loading buffer (control). Aliquots were stored at -20°C between gels and thawed completely at

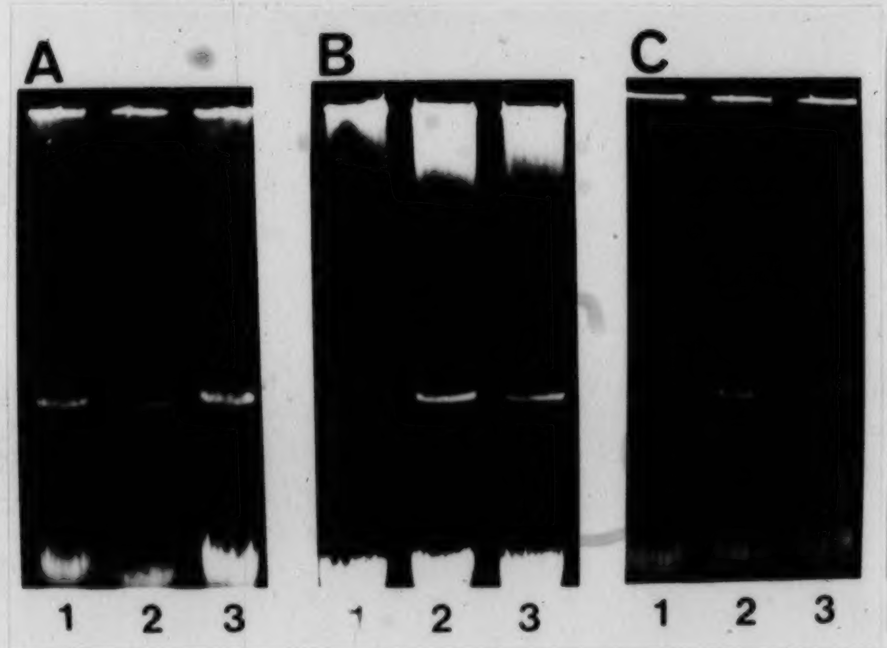

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Plate 5.7 Eclipse of *Flavobacterium* (BW-2) plasmids treated with Pronases or SDS and stored at -20°C for 0, 27 or 49 hr.

Panel A: no storage of isolated plasmids; Panel B: 27 hr storage; Panel C: 49 hr storage.
Lane 1: plasmids incubated with Pronases; Lane 2: plasmids incubated with SDS; Lane 3: untreated plasmids (control).

37°C before loading. Of the three plasmids observed at Time Zero (pF3, pF4 and pF5; Panel A, Plate 5.7), only the lower plasmid (pF5) remained after 27 hr storage (Panel B), yet all three plasmids were observed again after 49 hr storage (Panel C). Obviously, none of the treatments had any effect on the eclipse of the plasmids, compared with the untreated control preparation (lane 3).

Based on the preceeding results, it would have been easy to dismiss the theory of a protein:DNA complex being responsible for plasmid instability in *Flavobacterium* (8W-2) were it not for the following observations with reducing agents. At the suggestion of K.Roy (Dept. Microbiology, University of Alberta), the reducing agents β-mercaptoethanol (BME) or dithiothreitol (DTT) were incorporated into the plasmid preparations either as an added reagent during lysis and purification, or as an addition to the loading buffers. The presence of the reducing agents, especially BME, in some cases seemed to stabilize the plasmids, either delaying eclipse, or bringing the plasmids out of eclipse. An example of the effect of BME is shown in Plate 5.8. Aliquots of preparations with plasmids which had been observed to be in eclipse were either heated to 65°C for 10 min, or had BME added to the aliquots, or were simply thawed at room temperature (control). Panel A shows that only the aliquots receiving BME demonstrated plasmids. Another effect was noted in the same experiment when the samples, ± BME, were loaded into

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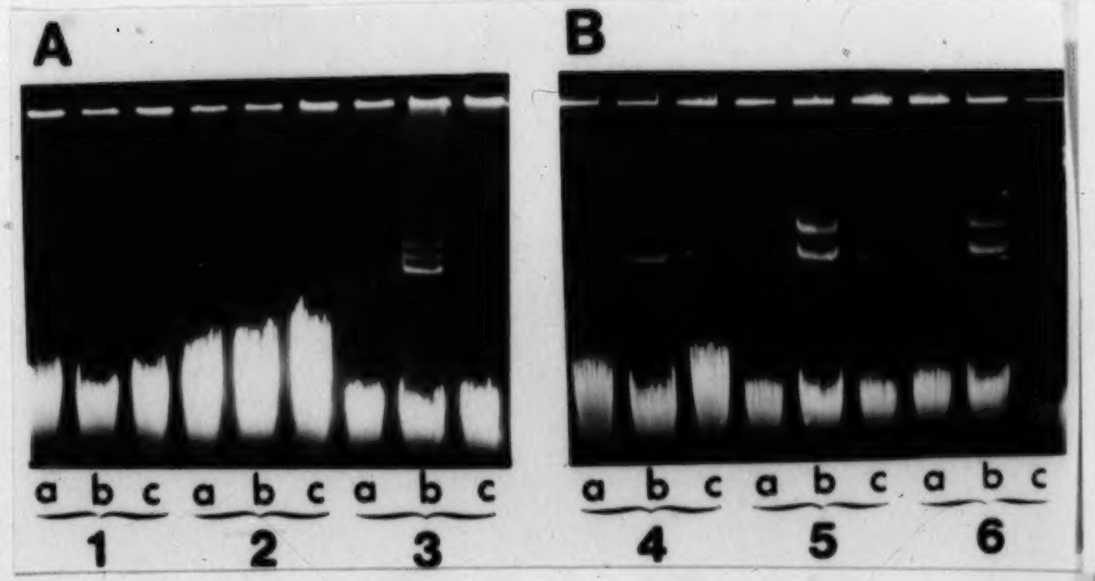

Plate 5.8 Effect of adding -mercaptoethanol to *Flavobacterium* (8W-2) plasmids in eclipse.
Panel A: agarose gel electrophoresed immediately after loading; Panel B: agarose gel electrophoresed approximately 30 min after loading.
Treatment "a": plasmids heated to 65°C for 10 min;
Treatment "b": β-mercaptoethanol added to plasmids;
Treatment "c": no treatment (control)
Lane 1: variant #46; Lane 2: variant pv41; Lane 3: variant Maint; Lane 4: variant pv17; Lane 5: variant pv4;
Lane 6: variant pv1.

the wells, but electrophoresis was delayed for approximately 1/2 hr. Panel B shows "diffusion cones" around the central wells of each replicate set. That is, the BME diffusing out of the central well to the adjacent samples brought the plasmids in those samples out of eclipse. This phenomenon is not merely an artifact of staining or visualization, since, when the entire gel was soaked in dilute BME overnight and was re-stained with EtBr and visualized with U.V. light, no additional plasmid bands were seen. It appears then, that BME (and DTT to a lesser extent) can act on plasmids in eclipse so that they become visible in agarose gels. This shows that, during eclipse, the plasmids are actually present in the aliquots, but somehow are not migrating during electrophoresis.

Of all the tests intended to demonstrate the presence of a protein associated with the *Flavobacterium* (8W-2) plasmids which made the plasmids "unstable", only BME showed results which differed from controls. The significance of the effect of BME is unknown, especially since it did not always prevent eclipse, nor did it always bring the plasmids out of eclipse. However, there is a possibility that the teritary structure of the plasmid is involved in the observed eclipse. That is, the plasmids are not inexplicably appearing and disappearing, but may be changing from CCC to OC forms, to exclude entry into the agarose gels; or are being linearized, thereby co-migrating with (and being obscured by) chromosomal material on standard 0.7% agarose gels (Chapter 3); or may be associating reversibly with contaminating biological materials such as proteins or RNA to change the structure or solubility of the plasmids.

Certainly, Hogrefe and Friedrich (1984) have demonstrated that there are tightly bound proteins associated with large plasmids from various lithotrophs. They also observed contamination of plasmid preparations with RNA, despite rigorous purification procedures. Miller and Kline (1979) have found plasmid:chromosome:RNA complexes which may also have associated proteins, and which were resistant to denaturation by proteinases and detergents. Rouviere-Yaniv and Yaniv (1979) demonstrated the presence of DNA-binding proteins in *E. coli*, similar to eukaryotic histones, which were involved in maintaining domains of supercoiling of plasmids. Relaxation of these domains resulted in numerous faint bands on agarose gels, related to the mobility of the single plasmid species as the supercoiled plasmid became progressively relaxed. This latter explanation may account for the multiple, faint bands observed occasionally on agarose gels of *Flavobacterium* (8W-2) plasmids (Plate 5.9) and in CsCl-EtBr gradients of *Flavobacterium* (8W-2) (data not presented). It is possible that these multiple bands represent the existing plasmid species in different stages of domain unfolding. Connors and Barnsley (1982) also have reported that two similar NAH plasmids demonstrated different degrees of supercoiling which influenced their relative mobilities in agarose gels.

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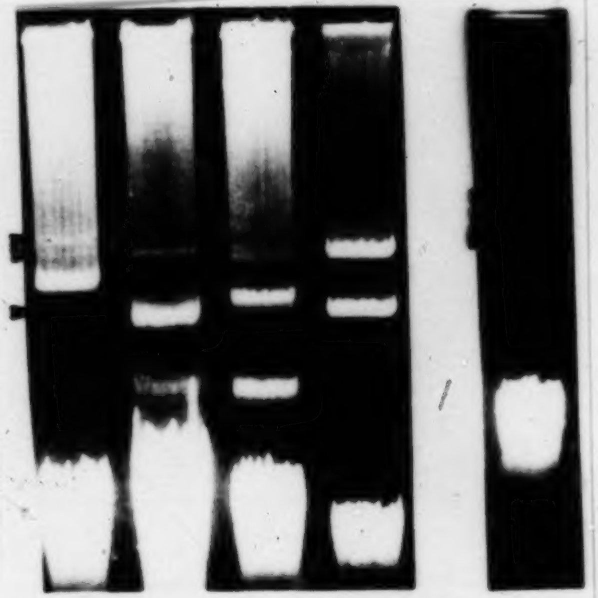

Plate 5.9 Multiple, faint plasmid bands (marked with arrows) observed in agarose gels with several *Flavobacterium* (8W-2) variants.

To explore the effect of tertiary plasmid structure, *Flavobacterium* (8W-2) plasmids in an agarose gel were deliberately nicked by exposure to EtBr and U.V. light, then re-electrophoresed in the presence of EtBr. Based on the relative mobilities of the three forms of plasmid (CCC, OC and linear), predictions can be made. For each original CCC band, nicking and electrophoresis should produce 2 or 3 bands: an upper band at the same position as the original CCC band, comprised of OC plasmid which does not migrate; a lower band comprised of un-nicked CCC DNA; and possibly a third band comprised of linear plasmid DNA which migrates more quickly than CCC DNA in the absence of EtBr (Mickel *et al.*, 1977), but more slowly in the presence of EtBr (Maniatis *et al.*, 1982). The presence and position of the last band is dependent upon the unlikely occurrence of a single double-stranded nick in the plasmid, and on the relative mobility as influenced by the presence of EtBr. The proportions and intensity of the different plasmid forms will be dependent upon the irradiation dose and degree of CCC nicking. The photograph shown in Plate 5.10 presents the results of the plasmid-nicking experiment. In the lower photographic plate the positions of the original bands are differentiated from the new bands appearing after re-electrophoresis. Lanes 1, 5, 8 and 11 contain a mixture of four CCC plasmid molecular weight markers which behaved as predicted, displaying a total of 8 bands after nicking, although not all bands were visible in all lanes. It is

Plate 5.10 Nicking of covalently closed circular (CCC) plasmid DNA by treatment with ethidium bromide and ultraviolet light.

Panel A: agarose gel immediately after electrophoresis and EtBr staining; Panel B: same agarose gel after exposure to U.V. light and re-electrophoresis. Positions of the original plasmid bands are marked with arrows.

Lanes 1,5,8,11: CCC marker plasmids (166Mdal, 112Mdal, 78Mdal, 33Mdal); Lane 2: T101; Lane 3: Maint; Lane 4: *Flavobacterium* (8W-2) wild type (overloaded); Lanes 6,7: *Flavobacterium* (8W-2) wild type; Lanes 9,10: plasmids from *Flavobacterium* (8W-2) grown in Fermenter "B" (Chapter 2).

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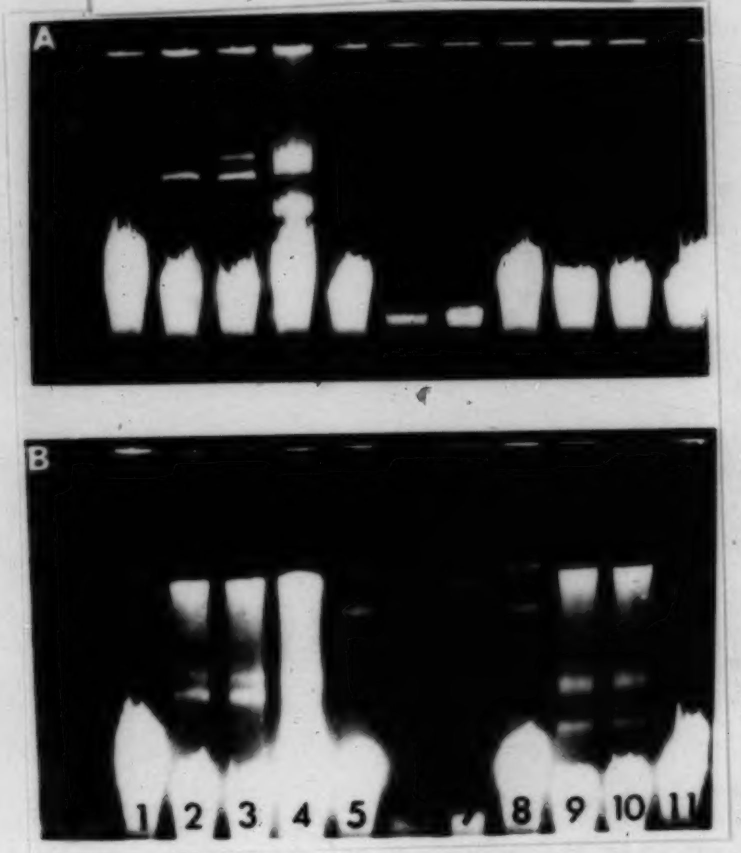
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assumed that nicking was incomplete because of the short irradiation time, and that, in the lower gel, the bands corresponding to the original bands represent the immobilized OC form of the plasmid, while the new bands represent the un-nicked CCC plasmids. The relative mobilities of the new bands were calculated and compared with the relative mobility of known (linear) chromosomal DNA in lanes 6 and 7, but no absolute relationships were apparent. Although the new bands in all cases had relative mobilities exceeding that of the linear chromosomal material, it is still likely that the new bands represent CCC DNA rather than linear DNA. This supposition may be strengthened by the fact that linear DNA intercalates more EtBr than CCC DNA, and this unwinding and charge neutralization lowers the relative mobility of the linear DNA (i.e. during the re-electrophoresis stage) by approximately 15% (Maniatis *et al*., 1982). Migration of CCC DNA, however, is increased above a critical concentration of EtBr (approximately 0.1 to 0.5µL/mL free EtBr; Maniatis *et al*., 1982), and the EtBr concentration in the staining solution was greater than 1µg/mL.

The plasmid pattern is more complex in the case of the *Flavobacterium* (8W-2) plasmids. Lanes 2 and 3 of Plate 5.10 which contain plasmids from variants T101 and Maint (pF1 and probably pF4) show only one original band (pF4) after re-electrophoresis. Unexpectedly, the upper original band (pF1) was not immobilized by exposure to EtBr and U.V., while the lower original band thickened considerably during re-electrophoresis and also migrated a very small distance. The two new bands migrated proportionally much further than any of the new bands of the molecular weight standards or the linear chromosomal DNA. This pattern would suggest that the *Flavobacterium* (8W-2) plasmids were influenced by the U.V. nicking process differently than the standard plasmids. Regarding the lack of immobilization of the upper original band, at least two explanations are possible. First, the upper band (pF1) may represent linear DNA, which would not be sensitive to the nicking process and therefore would not be immobilized. This explanation is less likely, because Mickel *et al*. (1977) demonstrated that linear DNA runs more quickly than CCC DNA, and no bands above the upper band (i.e. corresponding to the CCC form of the linear DNA) have been seen in these strains. A second possibility is that the upper plasmid (pF1) is not sensitive to U.V. nicking and remains in CCC form. The lower original band (probably pF4) must be sensitive to nicking, but the reason for the thickened band after electrophoresis and the smear in lanes 2 and 3 is unknown. The reason for the disproportionately large mobility of these plasmids upon re-electrophoresis is not known.

Similar results are seen with the wild type *Flavobacterium* (8W-2) plasmids (pF3, pF4, and pF5) in lanes 6, 7, 9 and 10. The upper (pF3) and lower (pF5) of the original plasmid bands disappear after re-electrophoresis, leaving a thickened middle band (pF4). Three new bands with the same spatial arrangement as the original bands appear at a distance proportional to the distance moved by bands in lanes 2 and 3, but disproportionately greater than the movement of the molecular weight markers and the linear chromosomal DNA. It appears that the upper and lower plasmids (pF3 and pF5) are not sensitive to U.V. nicking by the procedure used here, while the middle band (pF4) is sensitive. Again, the reason for the thickened middle band (pF4) and smearing after re-electrophoresis is not known.

Interpretation of the U.V. nicking experiment was complicated by the presence of multiple plasmid bands and by the unexpected observation that some plasmids were not immobilized by EtBr-U.V. treatment. Although no definite conclusions can be drawn regarding the tertiary structure or supercoiling of the *Flavobacterium* (8W-2) plasmids, it is apparent that the plasmids behave differently from *E. coli* and *Pseudomonas* spp. standard plasmids. Whether this unusual property is related to the phenomenon of eclipse is not known. It would appear that the plasmid bands seen during normal electrophoresis of *Flavobacterium* (8W-2) preparations are all CCC form, and that the appearance of the multiple, faint bands in some preparations (Plate 5.9) cannot be explained by linearization of the plasmids.

As previously discussed (Chapter 4), the TOL plasmid contains inverted repeats responsible for transposition and insertion/deletion events (Jeenes and Williams, 1981;

Lehrbach *et al.*, 1982). The presence of inverted repeats or similar transposition elements in the *Flavobacterium* (8W-2) plasmids has not been demonstrated; however, speculation about the existence of such elements could be used to explain a number of observed phenomena.

1. The multiple, faint bands (Plate 5.9) which were not adequately explained by progressive relaxation of supercoiling or by linearization of CCC plasmid DNA might be explained by multiple inserts mediated by inverted repeats. Such inserts, manifested as a "ladder" in agarose gels of the plasmid population, might in turn explain the observation of bright bands in *Flavobacterium* (8W-2) plasmid restriction digests (Plate 5.3).
2. The heterogeneous and shifting plasmid population in *Flavobacterium* (8W-2) (Chapter 4; Plate 4.3) might be the result of insertion/deletion or excision/integration phenomena mediated by inverted repeats. An interesting, although possibly misleading, observation is that the 4 largest plasmid species observed in *Flavobacterium* (8W-2) differ in size by increments of approximately 20 Mdal.
3. The instability of the metal resistance phenotype in *Flavobacterium* (8W-2) (Chapter 4) could be explained by transposition events (such as those recorded for the mercury resistance transposons; Silver and Misra, 1984).

4. The observed stability of the PAH$^+$ phenotype in the *Flavobacterium* (8W-2) population and difficulties encountered in attempted curing (Chapter 4) might occur if integration into the chromosome (or other plasmids) with subsequent excision promoted maintenance of degradative genes in the population.

5.3.4 Summary of Observations on the Plasmids of *Flavobacterium* (8W-2)

In summary, the nature of the *Flavobacterium* (8W-2) plasmids was elusive. They resisted separation into discrete plasmid species, and their unique "instability" prevented prolonged manipulative procedures. Restriction digests of the mixed plasmids were unsatisfactory. Several observed phenomena were not easily explained: the occasional presence of multiple, faint bands among the major plasmid bands on agarose gels or in CsCl-EtBr gradients; the unusual response to U.V. nicking; and, most puzzling, the eclipse of the plasmids.

The size of the *Flavobacterium* (8W-2) plasmids alone cannot account for this instability (although large plasmids are notoriously susceptible to shear forces) because the larger p38W-1 plasmid (and, indeed, all other plasmids studied) remained stable for long periods under identical storage conditions. As well, although irreversible disappearance of plasmids through *in vitro* instability has been reported (Wheatcroft and Williams, 1981), no reports of reversible disappearance have been found.

The possibility of the presence of a plasmid-associated protein was investigated, using the relaxation complexes of *E. coli* as a model. However, none of the results, except perhaps occasional observations with BME, and the presence of multiple, faint bands (such as those observed by Rouviere-Yaniv and Yaniv, 1979) concur with this theory. Transmission electron microscope studies were planned to look for plasmid-associated proteins, but were abandoned because of poor prognoses due to large plasmid size (D.Scraba, Dept. Biochemistry, University of Alberta; personal communication, 1984) and difficulties in obtaining sufficiently pure plasmids. In retrospect, it is unfortunate that no tests for RNA:plasmid associations were performed (e.g. by incubation with RN'ase). In fact, a combination of RNA and protein with *Flavobacterium* (8W-2) plasmids might explain restriction problems (as found by Hogrefe and Friedrich, 1984), the failure of ultracentrifugation as a preparative measure (as found by Miller and Kline, 1979), and possibly the phenomenon of eclipse through reversible conformational or solubility changes. The use of electrophoresis in 1.2% agarose should have been exploited more frequently also, to assess the presence of linear DNA.

It would be interesting to study in detail the plasmids of *Flavobacterium* (8W-2) subclone B-1L-25, since the plasmids in this strain alone were not observed to go into eclipse and also provided the best restriction results. It may also be significant that the 4 subclones of variant B-1L (including B-1L-25) were the only strains studied which demonstrated stable PAH phenotypes (discussed in section 4.3.5.2).

5.3.5 Conjugation and Transformation

Conjugation

No transconjugants were isolated using the first liquid medium conjugation method, which mated *Flavobacterium* (8W-2) with a *P. putida* (ATCC 33015) recipient. Of the ten Sm resistant putative transconjugants isolated in the second liquid medium mating trial, three had a yellowish pigment indicative of the parental *Flavobacterium* strain and therefore were discarded. The remaining putative transconjugants were tested for the presence of plasmids by the modified Casse technique, but no plasmids were observed. When two of the putative transconjugants were incubated with ASW + PBa, they did not demonstrate any aromatic degrading capability, and were therefore assumed to be spontaneous Sm resistant mutants of the recipient *Pseudomonas* DT82. No transconjugants were isolated using the solid medium mating technique. This inability to isolate transconjugants could be due to either to biochemical/genetic incompatabilities between the donor and recipient or to inadequate techniques.

It is likely that plasmid transfer did not occur, possibly because the liquid medium did not afford adequate contact between cells, or because the surface layer on

Flavobacterium (8W-2) (Chapter 2) prevented conjugation. It is not known whether many *Flavobacterium* spp. can conjugate either with isogenic recipients (not attempted in these studies) or with other genera (*Pseudomonas*, *Acinetobacter*, etc). Unfortunately, most conjugation procedures are designed for *E. coli* or *Pseudomonas* spp., and conjugation studies with *Flavobacterium* are currently under study in other laboratories (Saber and Crawford, 1985).

If transfer of plasmid(s) did occur in these experiments, several factors might have prevented isolation of the transconjugants. First, transconjugants might not have been detected since the choice of antibiotic and/or metal resistance markers was, in retrospect, unsuitable for consistently identifying PAH phenotypes (Chapter 4). Had an appropriate plasmid-borne marker with an easily detected phenotype been known, the chances of finding transconjugants would have been increased. Similarly, the implied chromosomal involvement in PAH degradation by *Flavobacterium* (8W-2) made the isolation of an aromatic-degrading transconjugant in a non-isogenic recipient unlikely. As well, had the plasmid(s) been transferred, incompatibility factors may have caused loss of the plasmid, especially in the *P. putida* (ATCC 33015) variant #13 which still contained a portion of the TOL plasmid. Had the plasmid(s) been transferred, stably maintained, and been capable of demonstrating an easily monitored phenotype, there is still the unknown factor of intrageneric expression of

Flavobacterium genes by the recipient genus.

Therefore, from these few experiments, it is not known whether conjugal transfer of *Flavobacterium* (8W-2) plasmids is possible, or whether successful transfer could be detected. The best experimental design would have included isogenic conjugation studies, using a completely cured, chromosomally marked recipient to obviate the aforementioned incompatibility, expression and chromosomal involvement issues. However, as previously discussed (Chapter 4), no completely cured strains were ever isolated, precluding the isogenic conjugation studies.

A number of other researchers have also encountered problems in attempting conjugal transfer of degradative plasmids: Connors and Barnsley (1982) were unable to detect transconjugants at a detectable frequency when mating certain NAH-containing *Pseudomonas putida* strains, and Kiyohara *et al.* (1983) were unsuccessful in isolating PHN transconjugants with *Beijerinckia* or with intrageneric conjugation (Kiyohara *et al.*, 1981).

Transformation

In the transformation studies attempted, no transformants were isolated: no colonies grew on the selective plates, and of the several hundred that appeared on the differential plates, none demonstrated an ability to degrade PAH. The factors adversely affecting conjugation experiments also could prevent isolation of transformants.

However, due to inadequate procedural controls, it is not possible to ascertain whether this failure was due to technical inadequacies or to inherent lack of competence in the recipient cells. Certainly, inability to completely cure recipient strains may have hindered recovery of transformants through surface exclusion/incompatibility phenomena. As well, there is the unknown effect of intrageneric expression of transformed genes and host cell effects such as envelope transport proteins (Shapiro *et al*., 1980). In addition, there are several other factors which may explain the lack of observed transformation: the large size of the plasmids; possible requirements for chromosomally encoded genes for complete expression of degradative functions; and marker choice.

In addition to these factors, there is the added concern that transformation of *Flavobacterium* spp. may not be possible with current techniques. For example, Negoro *et al*. (1980) found that the surface layer of *Flavobacterium* sp. K172 interfered with transformation and had to be removed enzymatically before transformation could occur. Even so, transformation was observed at a very low frequency of 11 transconjugants per 6×10^9 recipient cells. In addition, Beschle *et al*. (1984) found that none of the conventional protocols for transformation of *E. coli* and *Pseudomonas* spp. were successful with two *Flavobacterium* strains.

Therefore, it appears that transformation and conjugation experiments were hampered by a plethora of problems. The most important of these would seem to be lack of a suitable recipient (regarding expression and incompatibility) and lack of adequate screening techniques for detection of transformants and transconjugants.

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Chapter 6

Conclusions

6.1 Isolation, Taxonomy and Growth of Isolates

Crude oil-degrading mixed bacterial cultures proved to be good sources of pure isolates capable of metabolizing PAH. However, the capillary gas chromatographic analysis of residual oil which was used as a primary screen overestimated the degradative capability of the isolates. That is, a number of compounds whose GC peaks decreased during incubation with the isolates, implying metabolism, were subsequently shown to have been degraded only partially, or simply hydroxylated by the isolates. The results from sprayplates were often misleading due to technical reasons, and viable plate count results were sometimes questionable because of suspected bacterial adherence to the insoluble substrates. The best results showing bacterial metabolism of PAH were obtained using radiochemicals. In this way, mineralization of specifically labelled PAH could be quantitated by measuring $^{14}CO_2$ evolution, and partial metabolism could be observed by TLC with autoradiography. The usefulness of these techniques is severly restricted by the availability of radiochemicals.

It was found that the three chosen isolates' growth could be enhanced by the use of media supplements such as trypticase soy broth or yeast extract. Although it was originally believed that growth in selective media would be necessary for maintenance of the plasmids, they appeared to be stable *in vivo* during limited transfer in rich medium.

Generic identification of the PAH-degrading isolates was not definitive, partly because a minimum of taxonomic tests were performed, and partly because the nomenclature of environmental isolates is incomplete. This was especially true of *Flavobacterium* taxonomy. As well, identification of isolates on the basis of biochemical traits may have been complicated by the presence of catabolic plasmids.

The pure PAH-degrading isolates demonstrated a range of degradative capabilities and represented a number of genera, indicating that PAH degradation is not, as the literature would suggest, the exclusive domain of *Pseudomonas* spp.

6.2 Plasmid Isolation

The majority of published plasmid isolation techniques are designed for use with *E. coli*, or similar genera containing small plasmids; of the techniques designed for isolating degradative plasmids or larger plasmids, many are directed towards *Pseudomonas* species. This caused problems in isolating plasmids from the *Acinetobacter*, *Aeromonas* and *Flavobacterium* spp. described in this thesis. Modifications to the standard procedures were necessary in most cases, although the unmodified procedures often were adequate for the *E. coli* and *P. putida* controls. The most important factors in consistent plasmid isolation appeared to be: age of the cells; mass of the starting cell suspension; and lysis pH and NaCl salting out duration for separation of plasmid and chromosomal DNA. When these factors were optimized, it was found that all PAH-degrading isolates, except one, contained at least one plasmid. It is not known whether the high incidence of plasmid-containing isolates is significant with respect to environmental PAH degradation.

6.3 Generation and Characterization of Phenotypic Variants

Chemical, physical and biochemical curing techniques were unsuccessful in completely curing any of the three chosen isolates. This may have been due to technical inadequacies, especially regarding appropriate phenotypic screening methods (i.e. using indirect antibiotic- and metal-resistance), or to biochemical or genetic properties of the isolates. Regardless of incomplete curing, variants with altered PAH phenotypes were isolated and the nature of these variations was investigated by defining the PAH phenotype in more detail. Mineralization studies with radioactive substrates revealed that some variants had reduced degradative capabilities.

Spectrophotometric assays suggested that all variants were capable of the first, hydroxylative attack on specific PAH, even though further metabolism did not always follow. Several possible explanations for this common oxidative ability were suggested, including: the existence of redundant genes (chromosomal or plasmid-encoded) for degradative enzymes; or constitutive production of broad specificity oxygenases; or the presence of isofunctional but separately regulated degradative enzymes. The results presented in this thesis did not identify the correct explananation(s).

The accumulation of pathway intermediates by some variants of the genera studied provided information regarding degradative pathways (e.g. phenanthrene is degraded via o-phthalate; dibenzothiophene by a previously published pathway). Growth studies using minimal medium plus single carbon sources provided information about enzyme induction and regulation. The use of radioactive intermediates of degradative pathways would have complemented spectrophotometric studies, had the radioisotopes been available. It was inferred from TEM studies that some phenotypic variants may have arisen through changes in the cell envelope (e.g. surface "gum" production or permeability changes) rather than directly through alteration of degradative enzymes or regulatory genes, but proof of this supposition was outside the scope of the thesis.

Correlation of plasmid content with PAH phenotype was not possible with any of the three isolates chosen. Phenotypic variants of *Aeromonas* (38W-HC10-1) exhibited a single plasmid which co-migrated with the wild type plasmid and showed similar digest patterns. This lack of plasmid alteration might indicate that the degradative genes of *Aeromonas* (38W-HC10-1) are not plasmid-borne, but sufficient evidence to locate the genes on the chromosome is lacking.

Although phenotypic variants of *Acinetobacter* (8W-1) and *Flavobacterium* (8W-2) usually displayed plasmid profiles differing from the wild type, it was not possible to assign specific degradative functions to individual plasmids. In the case of *Acinetobacter* (8W-1), this was due in part to lack of information; with further experimentation, it is possible that plasmids could be correlated with the limited biphenyl phenotype. In the case of *Flavobacterium* (8W-2), several factors conspired to preclude the correlation of phenotype and plasmid content: the presence of multiple plasmids; the possibility of chromosomal involvement and presence of isofunctional enzymes; the extent of the phenotype; and the variability of the plasmid profile. It is likely that both plasmid and chromosomal genes are involved in PAH degradation by *Flavobacterium* (8W-2), as has been reported for naphthalene (NAH), phenol, toluene (TOL), phenylacetate and nicotinate (NIC), n-alkanes (OCT) and camphor (CAM).

6.4 Characterization of Putative Degradative Plasmids

Characterization of the single plasmid of *Aeromonas* (38W-HC10-1) was relatively easy, using centrifugation techniques for isolation. Restriction enzyme digest patterns and ^{32}P-hybridization studies showed no similarity to the TOL plasmid pWWO. However, this observation may not be significant because of the diversity of TOL plasmids.

Specific problems were encountered with *Flavobacterium* (8W-2) because of the presence of multiple plasmids. Techniques designed for separation of plasmid and chromosomal DNA (e.g. centrifugation) would not separate plasmid species, and secondary isolation techniques (e.g. recovery from agarose gels) had limited success. The appearance of a few bright bands in *Flavobacterium* (8W-2) total plasmid digests suggested the presence of repeated regions. These might be related to the speculated redundant genes, or may simply indicate that the multiple plasmids are related, being derivatives of a single plasmid.

No definite explanation for the unique *in vitro* instability of *Flavobacterium* (8W-2) plasmids was found, but possibilities include association of the plasmids with macromolecules (such as protein or RNA), or reversible changes in tertiary structure (such as relaxation or linearization). Further experimentation is required to resolve this question. The fact that the phenomenon was peculiar to the *Flavobacterium* strain is noteworthy.

It does not seem likely that completely cured isogenic recipients of *Flavobacterium* (8W-2) can be produced easily. This precludes simple "shotgun cloning" experiments with introduction of manipulated plasmids, or simple conjugation or transformation experiments. Therefore, it may be wisest to consider the use of techniques such as transposon mutagenesis to aid in future plasmid studies with *Flavobacterium* (8W-2). Future studies also would have to consider the suspected chromosomal influence of the host cell on PAH phenotype. Perhaps the use of broad host-range vectors such as RP64 could be exploited to overcome expression problems in intrageneric transfer of PAH plasmids.

In general, it is apparent from this thesis that genetic engineering of environmental isolates is difficult due to lack of suitable published genetic manipulation techniques such as plasmid isolation, conjugation and transformation procedures. Because the genera studied in these investigations, particularly the *Flavobacterium* sp., are genetically uncharacterized with respect to expression of genes, presence and specificity of transposons, existence of endogenous restriction endonucleases, and mechanisms of plasmid replication and transfer, the task of studying the role of plasmids in PAH degradation by many environmental bacteria will be hindered until basic genetic information for genera other than *Escherichia* and *Pseudomonas* becomes available. Alternatively, environmental *Pseudomonas* strains may be used for future genetic studies.

The probable involvement of the chromosome, whether through regulatory or structural genes, is another barrier to facile genetic engineering for PAH degradation: a suitable host with the appropriate chromosomal complement would be necessary for successful expression of introduced degradative plasmid genes. This might also include hosts with functions secondary to the degradative enzymes, such as outer membrane uptake proteins or specific permeability characteristics, both of which may be important for metabolism of insoluble PAH substrates.

Certainly, the development of genetically engineered isolates capable of PAH degradation is a complex task which cannot be accomplished quickly. This thesis has served to highlight some of the problems inherent in such an endeavor.

Appendix

Artificial Sea Water (ASW)

23.4 g NaCl
0.75 g KCl
7.0 g $MgSO_4 \cdot H_2O$
1 L distilled water
pH 7.3

Nitrogen, Phosphorus Supplement (NP)

420mL 10% K_2HPO_4
180mL 10% KH_2PO_4
60 g NH_4NO_3
pH 7.3 adjusted with 40% NaOH
filter sterilized

ASW + NP

ASW plus 1% v/v NP supplement

Basal Marine Agar (BMA)

1 g yeast extract; Difco
1 g proteose peptone #3; Difco
20 g purified agar; Difco
1 L ASW
pH 7.5 before autoclaving

Jobson's Medium

0.5 g K_2HPO_4
1.0 g NH_4Cl
2.0 g Na_2SO_4
2.0 g KNO_3
0.2 g $MgSO_4 \cdot 7H_2O$
trace $FeSO_4$
1 L distilled water

Anacker and Ordal Agar

0.05% tryptone; Difco
0.05% yeast extract; Difco
0.02% sodium acetate
0.02% beef extract; Difco
0.9% agar; Difco
pH 7.2 to 7.4

Salty Trypticase Soy Broth (STCS)

30 g dehydrated trypticase soy broth; BDH
23.4 g NaCl
1 L distilled water

Dilute STCS (1/5 Strength STCS)

6 g dehydrated trypticase soy broth; BDH
23.4 g NaCl
1 L distilled water

Salty Minimal Agar

4.4 g	K_2HPO_4
1.7 g	KH_2PO_4
2.1 g	NH_4Cl
23.4 g	NaCl
10mL	100X Salt Solution*
990mL	distilled water
20 g	purified agar; Difco

* 100X Salt Solution

19.5 g	$MgSO_4 \cdot 7H_2O$
5.0 g	$MnSO_4 \cdot H_2O$
5.0 g	$FeSO_4 \cdot 7H_2O$
1.0 g	ascorbic acid
1 L	distilled water

Glucose Oxidative/Fermentative Agar (O/F)

Solution A:

23.4 g	NaCl
0.75 g	KCl
7.0 g	$MgSO_4 \cdot 7H_2O$
1.0 g	yeast extract; Difco
0.03 g	bromthymol blue
15.0 g	purified agar; Difco
750mL	distilled water

Solution B:

10.0 g	glucose
200mL	distilled water

Solution C:

0.50 g	$NH_4H_2PO_4$
0.50 g	K_2HPO_4
50mL	distilled water

All solutions adjusted to pH 7.15 before autoclaving; Solutions A and B mixed after cooling, then Solution C added and mixed before dispensing medium.

Synthetic Hydrocarbon Mixture

160 mg/mL	hexachloroethane (marker)
40.6 mg/mL	1- methylnaphthalene
40.2 mg/mL	2,6-di methylnaphthalene
39.1 mg/mL	2.3-di methylnaphthalene
41.5 mg/mL	n-pentadecane
42.0 mg/mL	n-hexadecane
40.8 mg/mL	n-heptadecane
40.0 mg/mL	pristane
≤40 mg/mL	phenanthrene (saturated)
≤40 mg/mL	anthracene (saturated)

The above compounds were dissolved in benzene

Electrophoresis Loading Buffers

16% Ficoll 400,000; Pharmacia
50mM Tris
20mM Na_2EDTA
trace bromphenol blue or orange G

33% glycerol
7% SDS
0.07% bromthymol blue
in distilled water

Aromatic Fraction Preparation

Two millilitres of Prudhoe Bay crude oil were deasphaltened by diluting the oil with 5mL n-pentane. A 10cm long glass wool-packed liquid chromatography column was prepared with 2cm of benzene-washed sand on top of the packing material. The oil solution was applied to the top of the column and eluted with n-pentane (pesticide grade; Fisher), collecting the effluent until it was colorless. The effluent was dried overnight at room temperature in a forced air fume hood.

The deasphaltened oil was redissolved in 5mL n-pentane and applied to a freshly prepared liquid chromatography column. This column consisted of 10g activated silica gel (mesh 100-200, Davison Chemicals, MD; heated to 120°C for 3 hr to activate) overlaid with 10g alumina gel (mesh 70-230; Camag, West Germany) in pentane. The oil sample was rinsed into the column with two 5mL aliquots of n-pentane, then eluted with 30mL n-pentane. Forty-five millilitres of effluent were collected, which consisted of the saturate fraction of PB oil. This fraction was either discarded or retained for other studies. The top of the column was then rinsed with two 5mL volumes of benzene (pesticide grade; Fisher), and the aromatic fraction was eluted with 90mL of benzene. The collected eluent was evaporated to near dryness in a rotary evaporator (Roto-Vap; Buchler, West Germany) or in a glass concentrator (Kontes, NJ). The residue was redissolved in benzene to the original volume (2mL).

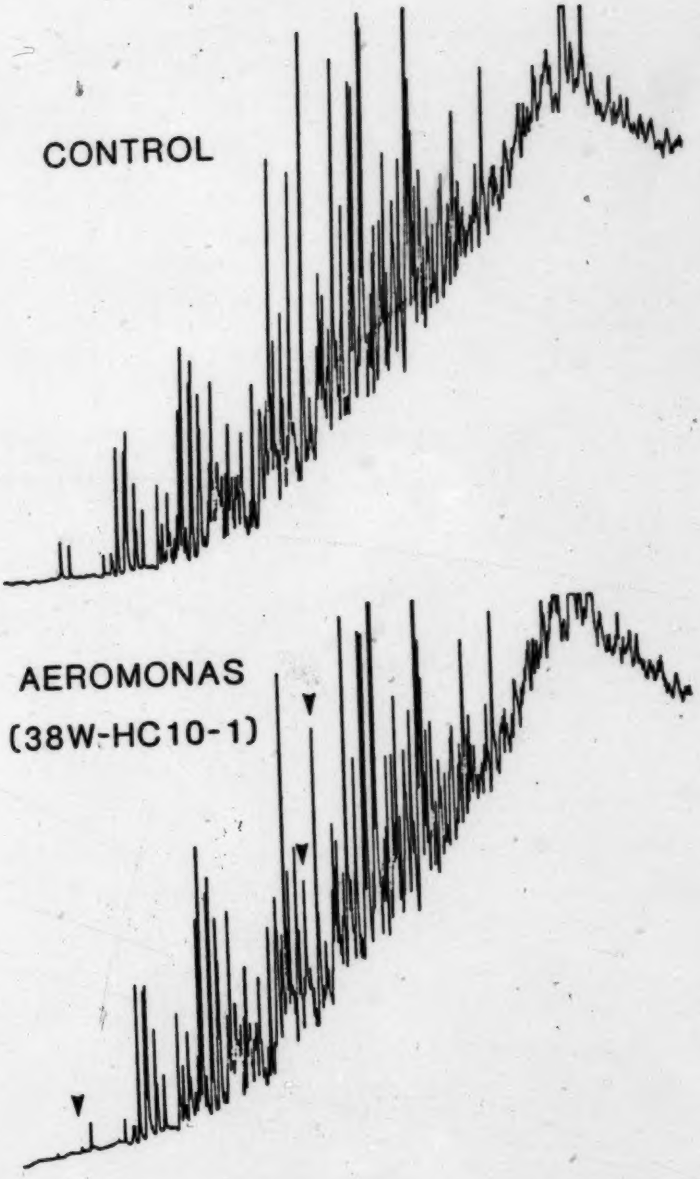

Figure A1. Capillary gas chromatograms of the aromatic fraction of PB crude oil incubated with *Aeromonas* (38W-HC10-1) or with uninoculated medium (control). Chromatograms are directly comparable. Arrows indicate reduction in peak height.

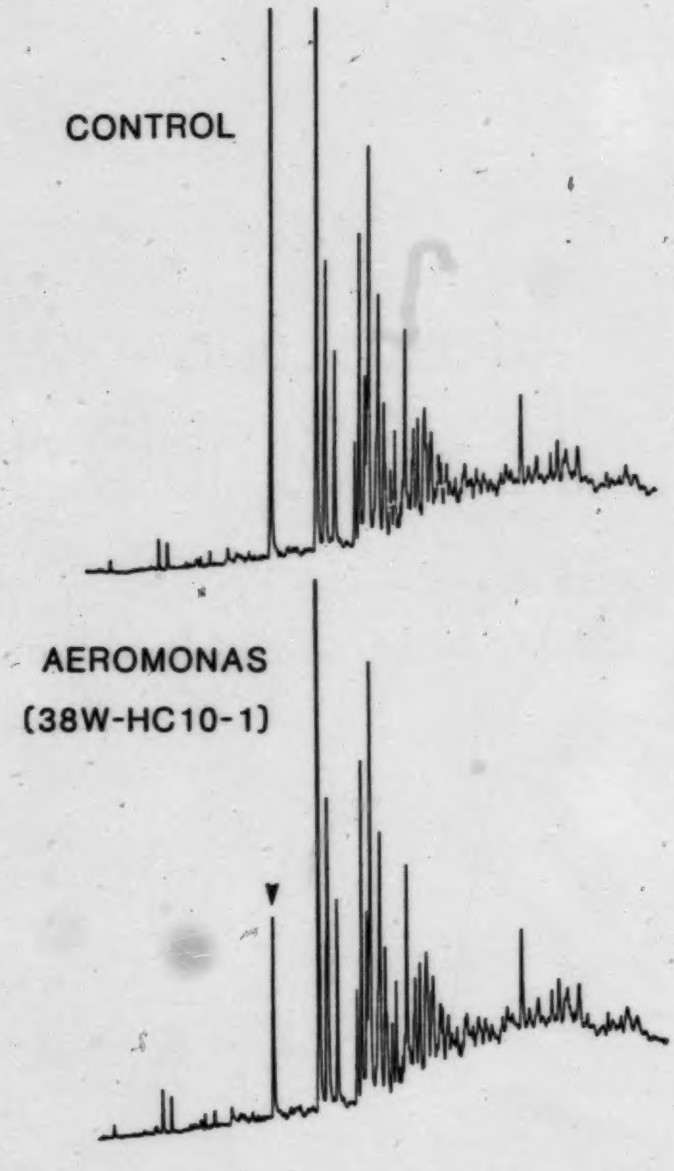

Figure A2. Capillary gas chromatograms of the sulfur heterocycles of PB crude oil incubated with *Aeromonas* (38W-HC10-1) or with uninoculated medium (control). Chromatograms are directly comparable. Arrows indicate reduction in peak height.